Sex steroid binding proteins in fish

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DECLARATION

I hereby declare that this Thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis, and to the best of my knowledge and belief no material previously published or written by another person except when due acknowledgement is made in the text of the Thesis.

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Abstract

A sex steroid binding protein (SBP) binding E_2 with high affinity has been detected in the pleuronectid greenback flounder (*Rhombosolea tapirina*), the sparids black bream (*Acanthopagrus butcheri*) and snapper (*Pagrus auratus*), and its presence has been confirmed in the salmonid rainbow trout (*Oncorhynchus mykiss*). SBP binding characteristics were measured using a hot saturation assay for trout, bream and snapper and a cold saturation assay for flounder.. Bound and unbound steroid were separated by incubation with dextran-coated charcoal (DCC). Affinity for E_2 was highest in trout (k_D = 0.44 nM), followed by bream (k_D = 3.39 nM) and snapper (k_D = 10.7 nM). The lowest affinity was found in flounder (k_D = 84.7 nM). Binding capacity, however, was greatest in flounder (B_{max} = 164 nM), followed by trout (B_{max} = 92 nM), and then bream and snapper with B_{max} = 50 and 39 nM respectively. Binding of E_2 to SBP had a very rapid rate of association, and most dissociation occurred within 5 min.

To confirm that the plasma protein measured here was SBP, the relative binding affinities of SBP for a range of steroids was measured. In trout, bream and snapper, SBP bound E_2 with the highest affinity, followed by T. In contrast, flounder SBP bound T more than twice as strongly as E_2. The rank orders of affinity of binding indicate the importance of an unhindered 17β-hydroxyl group, and a 3-hydroxyl or 3-ketone group for high affinity binding to SBP. These requirements for high affinity binding are present in most animals possessing SBP and indicate conservation of the SBP molecule through evolution.

The presence of seasonal changes in SBP binding characteristics was investigated in female trout, bream and flounder. The binding capacity of trout and bream SBP was significantly greater in vitellogenic than non-reproductive stage fish. A decrease in binding affinity was found in male trout injected with E_2 compared to control fish. This difference was removed by partial purification of SBP by gel filtration, and may have resulted from competitive inhibition of E_2 binding by vitellogenin. No differences in flounder SBP were found.

The effect of short term confinement stress on SBP binding characteristics was examined in female bream, and trout. Confinement of bream for 6 h (but not 1 or 24 h) after capture significantly reduced the binding capacity of SBP. No significant differences in binding characteristics were detected in trout after 5 h confinement, or at 3 and 6 h after treatment with either cortisol or saline, although binding capacity tended to be lower in fish treated with cortisol rather than saline at 6 h post-injection. Relative binding studies indicated that plasma cortisol at concentrations 100x or more greater than plasma E_2 may displace E_2 from SBP in bream. Physiological levels of cortisol did not displace E_2 from SBP in trout.

The results presented here predominantly support the idea of SBP as a steroid reservoir, protecting and transporting E_2 and/or T to target tissues. However, binding characteristics of SBP differ between species and changes of SBP with reproductive development are not consistent. This suggests that the either the role of SBP in reproduction may not be profound, it may be important in a way not investigated in the present study, or SBP may have a role that differs in some way between different species.

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1. General Introduction

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The endocrine system controls reproduction through the hypothalamic-pituitary-gonad axis (reviewed by Pankhurst, 1998). Gonadotropin-releasing hormone (GnRH) stimulates release of gonadotropins (GtH) from the pituitary. In response to GtH, the ovary produces the gonadal steroids 17β-estradiol (E_2) and testosterone (T). T is a precursor for E_2 synthesis, and hepatic production of the yolk precursor vitellogenin (VtG) is stimulated by binding of E_2 to specific receptors in liver cells. Uptake of VtG by growing oocytes is then mediated by GtH. E_2 and T also have feedback effects at the level of the brain and pituitary.

Hormone messengers which must travel from the secretory organ to the target tissue are transported in the blood in either a free state, or bound to proteins. Sex steroid binding protein (SBP) (also known as sex hormone binding globulin (SHBG) and testosterone estradiol binding globulin (TeBG)) binds the reproductive steroid hormones T and E_2 with high affinity in the plasma of many vertebrates (reviewed in Joseph, 1994). These steroids also circulate bound with low affinity in mammals to albumin (Westphal, 1986), the predominant functions of which are osmotic regulation, and transport of free fatty acids (Kragh-Hansen, 1981). Corticosteroids bind to a corticosteroid binding globulin (CBG), and progesterone binds to a progesterone binding globulin (PBG) in some species (reviewed in Westphal, 1986). An androgen binding protein (ABP) is also present in the testes of some animals (reviewed in Joseph, 1994). SBP has not been found in some vertebrate species including pigs, guinea pigs, horses, donkeys, camels, adult rats and mice and birds (reviewed in Westphal, 1986; Petra, 1991; Joseph, 1994). It has been suggested, however, that the lack of SBP in some of these species may reflect a lack of sensitivity of some methods used to measure the presence of SBP (Joseph, 1994). The absence of SBP from these species may suggest that the presence of SBP may not be essential. However, there is also the possibility that essential functions of SBP may performed by other proteins in species lacking SBP.

SBP in humans is a large glycoprotein with a molecular weight of about 90 kDa, a carbohydrate content of around 12 %, 2 subunits and a single binding site (reviewed in Hammond, 1990; Hammond, 1995). It is generally believed that SBP transports

biologically important steroids in the plasma, regulating their clearance, and availability at target tissues (Hammond, 1995). The metabolic clearance rate of T for example, is proportionately reduced in the presence of SBP (Stanczyk *et al.*, 1986). Classically, protein-bound steroids have been viewed as biologically inactive, and the presence of SBP has been considered to enable high concentrations of steroids to be transported in a controlled manner, and provide an easily accessible pool of hormone (Siiteri *et al.*, 1982; Westphal, 1986). Secretion of hormone from gonadal tissue into the blood is also increased by the presence of binding proteins. Removal of the steroid from the cell membrane by binding proteins maintains a concentration gradient for continued diffusion of the steroid out of the cell (Westphal, 1986).

Levels of SBP in humans have been found to increase in response to endogenous and exogenous increases in E_2, and decrease in response to increased levels of T. This led to a proposal by Burke and Anderson (1972) that changes in SBP in response to changing plasma E_2 and T concentrations, and differential binding of these steroids to SBP act as a servo-mechanism. Increased plasma E_2 increases the SBP concentration and acts to further increase the ratio of unbound E_2 to unbound T, as T in humans is bound with higher affinity than E_2. An increase in T decreases SBP and increases the ratio of unbound T to unbound E_2. However, this theory assumes that unbound steroid is more important than bound steroid (Burke, 1977), and that tissues respond to the ratios of T and E_2 rather than absolute levels of the steroids (Rosner, 1990).

The classical view of steroid action is that of steroids entering target cells by diffusion and binding to cytoplasmic or nuclear receptors resulting in a genomic response (Lazier *et al.* 1987). However, it is now established that in addition to this, steroids can also cause a non-genomic intracellular response by binding to membrane receptors (Thomas, 2000). It is thought that free diffusion allows sufficient steroid to enter cells to induce the genomic biological response (reviewed in Westphal, 1986), however, some authors disagree (eg. Lobl, 1981; Siiteri *et al.*, 1982). The experimental inconsistencies between steroid concentrations required for maximal receptor response and measured free steroid concentrations are partially resolved if the albumin-bound fraction is considered to also be able to diffuse into cells (Pardridge and Mietus, 1979a, b; Siiteri *et al.*, 1982; Manni *et al.* 1985). However, it has been suggested that both free and albumin bound steroids are

irreversibly cleared from the circulation (Siiteri *et al.*, 1982), questioning the relative importance of the free and SBP-bound fractions. Lobl (1981) has postulated that active transport of steroid across the target cell membrane by a mechanism involving the binding protein and cell surface receptors would allow much greater specificity by the target cell than a free diffusion mechanism. In support of this, cell membrane receptors specific for SBP have been found on several tissue membranes. For example, SBP bound to E_2 binds to plasma membranes of human decidual endometrium (Strel'chyonok *et al.*, 1984). However, SBP is required to be unliganded to bind to a receptor on human prostatic tissue, and the SBP-receptor complex is then able to bind steroids (Hryb *et al.*, 1990). This activates a second messenger system involving intracellular accumulation of cAMP (reviewed in Rosner *et al.*, 1992). Binding of SBP to receptors on human breast cancer cells, however, led to internalization of SBP by receptor-mediated endocytosis (Porto *et al.*, 1991). Other authors question whether intracellular SBP found in a number of tissues (eg. monkey testes, liver and adrenal cortex; Bordin and Petra, 1980) originates in the plasma, suggesting instead *in situ* synthesis of the protein (Baulieu, 1986).

Plasma proteins with the characteristics of SBP, namely high affinity, low capacity binding to T and E_2 and low affinity for other steroids, have been found in vertebrates from a wide phylogeny. Early studies concentrated on SBP in humans (reviewed by Westphal, 1986) and then other mammals including marsupials, hedgehogs, bats, lagomorphs, and non-human primates (reviewed in Westphal, 1986). The presence of SBP in a range of non-mammalian vertebrates including cyclostomes, elasmobranch and teleost fishes, amphibians, and reptiles has also been established (reviewed by Wingfield, 1980).

In teleost fishes, plasma binding of E_2 and/or T has been demonstrated in a number of species including rainbow trout *Salmo gairdneri* = *Oncorhynchus mykiss* (Fostier and Breton, 1975), goldfish *Carassius auratus* (Pasmanik and Callard, 1986), spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1994), Atlantic salmon *Salmo salar* (Lazier *et al.*, 1985), common carp *Cyprinus carpio* (Chang and Lee, 1992), brown trout *Salmo trutta* (Pottinger, 1988), brook char *Salvelinus fontinalis* (McPherson *et al.*, 1988), African catfish *Clarius gariepinus* (Rebers *et al.*, 1991) and the winter flounder *Pseudopleuronectes americanus* (Sloop *et al.*, 1984). Molecular weights of teleost SBP are only available for 3 species. The estimated molecular weights are 64 kDa for SBP in eel

Anguilla japonica (Chang *et al.*, 1994), around 135 kDa in spotted seatrout (Laidley and Thomas, 1994) and 194 kDa in carp (Chang and Lee, 1992). Binding affinities in teleosts range from a k_D of about 2 nM in goldfish (Pasmanik and Callard, 1986; Van Der Kraak and Biddiscombe, 1999) to a k_D of 48 nM in brown trout (Pottinger, 1986). Binding capacity tends to range from 100 to 400 nM in the majority of species studied (eg. Pasmanik and Callard, 1986; Pottinger, 1988; Foucher *et al.*, 1992; Laidley and Thomas, 1994; Van Der Kraak and Biddiscombe, 1999). The function of SBP in non-mammalian vertebrates is assumed to be the same as in mammals, ie. protection of steroids from metabolism and excretion (Callard and Callard, 1987). As in mammals, E_2 administration in rainbow trout was found to increase SBP concentration, but in contrast to mammals, T administration had no effect (Foucher *et al.*, 1991). These last authors also showed production of SBP in hepatocyte culture, and found that growth hormone appeared to maintain basal SBP levels.

In the present study, characteristics of SBP-steroid interactions were investigated in females of four teleost fish species: rainbow trout (*O. mykiss* Walbaum, Salmonidae), greenback flounder (*Rhombosolea tapirina* Günther, Pleuronectidae), black bream (*Acanthopagrus butcheri* Munro, Sparidae) and snapper (= red sea bream, *Pagrus auratus* Bloch & Schneider, Sparidae). Initially a method to measure SBP in goldfish (Van Der Kraak and Biddiscombe, 1999) was optimised for these species (Chapter 2, this volume) and the presence of SBP in these species was determined.

Peak levels of reproductive steroids can vary widely among teleost species. Rainbow trout mature during an annual migration to spawning grounds, and then release gametes in a single spawning event. High levels of E_2 and T present in the plasma of female fish are most likely due to the group synchronous development of the ovary. Peak concentrations of E_2 and T vary between populations, but may reach as high as 50 and 200 $ng.ml^{-1}$ for E_2 and T respectively (Scott *et al.*, 1980). In contrast, peak steroid levels are low in many marine species, especially those that display repeat spawning during the reproductive season (Pankhurst and Carragher, 1991). For example, the sparids black bream and snapper are repeat spawners with a daily cycle, and peak E_2 concentrations are about 4 and 3 $ng.ml^{-1}$ for bream and snapper respectively (Haddy and Pankhurst, 1998; Hobby and Pankhurst, 1997). Levels of T in these fish are similarly low (about 3 $ng.ml^{-1}$ for bream;

Haddy and Pankhurst, 1998, and less than 1 $ng.ml^{-1}$ for snapper; Hobby and Pankhurst, 1997). Greenback flounder have an extended spawning season during which there is repeat spawning by individuals which may be daily on an intermittent basis (Barnett and Pankhurst, 1999). Levels of steroids in flounder peak at low to moderate concentrations of 3 and 8 $ng.ml^{-1}$ for E_2 and T respectively (Barnett and Pankhurst, 1999). While it has been established that SBP levels vary between species, comparisons between different studies where different methods have been utilised are difficult. It was therefore important to use the same method to investigate SBP characteristics in several species with peak steroid levels ranging from high to relatively low. To establish whether SBP binding characteristics differed between these species in a manner related to the differing peak levels of reproductive steroids, the equilibrium dissociation constant (k_D) as a measure of binding affinity, and the binding capacity (B_{max}) were measured and compared (Chapter 3, this volume). To confirm the protein measured in the present study was SBP, the relative affinity of the protein for a range of C_{18}, C_{19} and C_{21} steroids was established (Chapter 4, this volume).

Cyclic changes in SBP levels have been investigated in several studies. However, while SBP changes in relation to mammalian reproductive cycles are well established, there is less information regarding non-mammalian vertebrates. In male teleosts, SBP concentrations may be constant throughout the annual cycle as in common carp (Chang and Chen, 1990). In contrast, there is a trend for a decline in SBP binding capacity during the spawning season in male salmonids (Pottinger, 1988; Foucher *et al.*, 1992). The results for female teleosts are similarly inconclusive. No seasonal or sex differences were found in k_D or B_{max} in goldfish (Pasmanik and Callard, 1986), and there was no correlation between plasma E_2 or T and B_{max} in female common carp (Chang and Chen, 1991). However, B_{max} and k_D in spotted seatrout both peaked around the stage of final oocyte maturation (Laidley and Thomas, 1997). To determine whether SBP levels changed in relation to reproductive status within a species, binding characteristics were measured in 3 species, rainbow trout, black bream and greenback flounder. The k_D and B_{max} of SBP were measured in non-reproductive fish (lowest steroid levels during the annual cycle), and fish undergoing vitellogenesis (generally the highest E_2 and T concentrations during the reproductive cycle) (Chapter 5, this volume).

Stress has an inhibitory effect on reproductive processes in fish. Circulating levels of T and E_2 may be reduced and the incidence of ovarian atresia increased (Pankhurst and Van Der Kraak, 1997). Effects may extend to a shutdown of reproductive processes for the current season (Carragher and Pankhurst, 1991), or impact on survival of progeny (Campbell *et al.*, 1992, 1994). The general assumption is that effects are exercised on the endocrine cascade, and probably through an effect of cortisol (Pankhurst and Van Der Kraak, 1997, 2000). However, less consideration has been given to post-secretion events including metabolism and clearance of steroid hormones from the plasma. Cortisol may have an effect through binding to SBP, reducing protection and thus increasing metabolism of sex steroids in the plasma, interfering with concentration of steroid at the receptors. The relative affinity of SBP for cortisol has been low in all species so far examined, with cortisol generally being one of the least effective competitors of those investigated. For example, a concentration of cortisol in excess of 1000 times the concentration of labelled steroid is required to displace half of the bound labelled steroid in spotted seatrout (Laidley and Thomas, 1994). However, in periods of stress, cortisol levels in plasma have been measured at much higher concentrations than those of the reproductive steroids. Plasma cortisol levels in wild snapper were 46 $ng.ml^{-1}$ after 4 days of confinement and handling while levels of E_2 were below the detection limit of the assay (~0.05 $ng.ml^{-1}$), ie. in this case the concentration of cortisol is around 1000 times greater than that of E_2 (Carragher and Pankhurst, 1991). To investigate the presence of a stress effect on SBP, binding characteristics of SBP in rainbow trout and black bream were investigated before and after stress. The relative affinity of SBP for cortisol was also measured to determine whether competition between E_2/T and cortisol for SBP binding sites could occur at physiological levels of these steroids (Chapter 6, this volume).

As results from Chapters 2 to 6 have been or will be published, there is some planned overlap of the General Introduction with the Introduction section for each chapter.

Preliminary work from Chapter 2 appears in the conference proceedings from the XIIIth International Congress of Comparative Endocrinology, Yokohama (Japan), November 16-21, 1997.

Hobby, A.C., and Pankhurst, N.W. (1997). Comparative aspects of steroid binding protein (SBP) in four fishes: a salmonid, a pleuronectid and two sparids. *In* "Advances in Comparative Endocrinology" Vol. 2 (S. Kawashima, and S. Kikuyama, Eds.), pp. 1369-1373. Monduzzi Editore, Bologna.

Material from Chapters 2, 3 and 4 appears in:

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A condensed version of Chapter 4 also appears in the conference proceedings from the 6th International Symposium on Reproductive Physiology of Fish, Bergen (Norway), July 4-9, 1999.

Hobby, A.C., Pankhurst, N.W., and Geraghty, D.P. (2000). Relative binding affinities of steroids for sex steroid binding protein (SBP) are similar despite differences between species in affinity and capacity for estradiol. *In* "Reproductive Physiology of Fish 1999" (B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Anderson, and S.O. Stefansson, Eds.) pp. 177. John Grieg A/S, Bergen, Norway.

Chapter 5:

Hobby, A.C., Pankhurst, N.W., and Geraghty, D.P. Differences in binding characteristics of sex steroid binding protein (SBP) between reproductive and non-reproductive female rainbow trout (*Oncorhynchus mykiss*), black bream (*Acanthopagrus butcheri*) and greenback flounder (*Rhombosolea tapirina*). *General and Comparative Endocrinology*, under review.

Chapter 6:

Hobby, A.C., Pankhurst, N.W., and Haddy, J.A. (2000). The effects of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology* **125A**, 85-94.

1.1 References

Barnett, C.W., and Pankhurst, N.W. (1999). Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther 1862). *Mar. Freshwater Res.* **50**, 35-42.

Baulieu, E.E. (1986). Steroid hormone binding proteins and their intra- and extra-cellular congeners. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 1-11. John Libbey Eurotext, London.

Bordin, S., and Petra, P.H. (1980). Immunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey *Macaca nemestrina*. *Proc. Natl. Acad. Sci. USA* **77**, 5678-5682.

Burke, C.W. (1977). Clinical significance of steroid-binding proteins in plasma. *In* "Endocrinology" (V.H.T. James, Ed.), pp. 463-466. Excerpta Medica, Amsterdam-Oxford.

Burke, C.W., and Anderson, D.C. (1972). Interrelationships of unbound testosterone and oestradiol in human serum at 37°C, and a biological role for sex-hormone-binding globulin. *Endocrinology* **53**, XXVI-XXVII.

Callard, I.P., and Callard, G.V. (1987). Sex steroid receptors and non-receptor binding proteins. *In* "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D.O. Norris, and R.E. Jones, Eds.), pp. 355-384. Plenum Press, New York.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biol. Reprod.* **47**, 1140-1150.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* **120**, 151-169.

Carragher, J.F., and Pankhurst, N.W. (1991). Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). *In* "Reproductive Physiology of Fish 1991" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 253-255. FishSymp91, Sheffield.

Chang, C.-F., and Chen, M.-R. (1990). Fluctuations in sex steroids and sex steroid-binding protein during the development and annual cycle of the male common carp, *Cyprinus carpio*. *Comp. Biochem. Physiol.* **97A**, 565-568.

Chang, C.F., and Chen, M.R. (1991). Lack of correlation found between plasma sex steroids and sex steroid-binding capacity during the annual cycle of the female common carp. *Bull. Inst. Zool., Academia Sinica* **30**, 81-86.

Chang, C.F., and Lee, Y.H. (1992). Purification of the sex steroid-binding protein from common carp (*Cyprinus carpio*) plasma. *Comp. Biochem. Physiol.* **101B**, 587-590.

Chang, C.F., Lee, Y.H., Yoshida, T., and Sun, L.T. (1994). Characterization of the plasma sex steroid-binding protein in eel (*Anguilla japonica*). *Comp. Biochem. Physiol.* **108B**, 189-197.

Fostier, A., and Breton, B. (1975). Binding of steroids by plasma of a teleost: the rainbow trout, *Salmo gairdnerii*. *J. Steroid Biochem.* **6**, 345-351.

Foucher, J.L., Niu, P.D., Mourot, B., Vaillant, C., and Le Gac, F. (1991). *In vivo* and *in vitro* studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Molec. Biol.* **39**, 975-986.

Foucher, J.L., Le Bail, P.-Y., and Le Gac, F. (1992). Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **85**, 101-110.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Hammond, G.L. (1990). Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocr. Rev.* **11**, 65-79.

Hammond, G.L. (1995). Potential functions of plasma steroid-binding proteins. *Trends Endocrinol. Metab.* **6**, 298-304.

Hobby, A.C., and Pankhurst, N.W. (1997). The relationship between plasma and ovarian levels of gonadal steroids in the repeat spawning marine fishes *Pagrus auratus*

(Sparidae) and *Chromis dispilus* (Pomacentridae). *Fish Physiol. Biochem.* **16**, 65-75.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048-6054.

Joseph, D.R. (1994). Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197-280.

Kragh-Hansen, U. (1981). Molecular aspects of ligand binding to serum albumin. *Pharm. Rev.* **33**, 17-53.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Laidley, C.W., and Thomas, P. (1997). Changes in plasma sex steroid-binding protein levels associated with ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **56**, 931-937.

Lazier, C.B., Lonergan, K., and Mommsen, T.P. (1985). Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. *Gen. Comp. Endocrinol.* **57**, 234-245.

Lazier, C.B., Mann, M., and Mommsen, T.P. (1987). Estrogen receptors and regulation of vitellogenesis. *In* "Reproductive Physiology of Fish 1987" (D.R. Idler, L.W. Crim, and J.M. Walsh, Eds.), pp. 178-182. Memorial University of Newfoundland, St. Johns.

Lobl, T.J. (1981). Androgen transport proteins: physical properties, hormonal regulation, and possible mechanism of TeBG and ABP action. *Arch. Androl.* **7**, 133-151.

Manni, A., Pardridge, W.M., Cefalu, W., Nisula, B.C., Bardin, C.W., Santner, S.J., and Santen, R.J. (1985). Bioavailability of albumin-bound testosterone. *J.Clin. Endocrinol. Metab.* **61**, 705-710.

McPherson, R., Hannum, J., and Greco, T. (1988). An investigation of an estrogen-binding component in the liver and plasma of brook char, *Salvelinus fontinalis*. *Comp. Biochem. Physiol.* **89A**, 615-619.

Pankhurst, N.W. (1998). Reproduction. *In* "Biology of Farmed Fish" (K.D. Black, and A.D. Pickering, Eds.), pp. 1-26. Academic Press, Sheffield.

Pankhurst, N.W., and Carragher, J.F. (1991). Seasonal endocrine cycles in marine teleosts. *In* "Reproductive Physiology of Fish 1991" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 131-135. FishSymp91, Sheffield.

Pankhurst, N.W., and Van Der Kraak, G. (1997). Effects of stress on reproduction and growth of fish. *In* "Fish Stress and Health in Aquaculture" (G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, Eds.), pp. 73-93. Cambridge University Press, Cambridge.

Pankhurst, N.W., and Van Der Kraak, G. (2000). Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout *in vivo*, through the action of cortisol. *Gen. Comp. Endocrinol.* in press.

Pardridge, W.M., and Mietus, L.J. (1979a). Transport of protein-bound steroid hormones into liver *in vivo*. *Am.J.Physiol.* **237**, E367-E372.

Pardridge, W.M., and Mietus, L.J. (1979b). Transport of steroid hormones through the rat blood-brain barrier. Primary role of albumin-bound hormone. *J. Clin. Invest.* **64**, 145-154.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Molec. Biol.* **40**, 735-753.

Porto, C.S., Gunsalus, G.L., Bardin, C.W., Phillips, D.M., and Musto, N.A. (1991). Receptor-mediated endocytosis of an extracellular steroid-binding protein (TeBG) in MCF-7 human breast cancer cells. *Endocrinology* **129**, 436-445.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1988). Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **70**, 334-344.

Rebers, F.E.M., Schulz, R.W., and Goos, H.J.T. (1991). Plasma sex steroid binding activity in the African catfish, *Clarius gariepinus*. *In* "Reproductive Physiology of Fish" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 129. FishSymp 91, Sheffield.

Rosner, W. (1990). The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocr. Rev.* **11**, 80-91.

Rosner, W., Hryb, D.J., Khan, M.S., Nakhla, A.M., and Romas, N.A. (1992). Sex hormone-binding globulin: Binding to cell membranes and generation of a second messenger. *J. Androl.* **13**, 101-106.

Scott, A.P., Bye, V.J., and Baynes, S.M. (1980). Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson). *J. Fish Biol.* **17**, 587-592.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., and Kuhn, R.W. (1982). The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38**, 457-510.

Sloop, T.C., Sherman, B., and Lucier, G.W. (1984). Characterization of a cytosolic steroid-binding protein in the liver of the winter flounder, *Pseudopleuronectes americanus*. *Gen. Comp. Endocrinol.* **55**, 157-162.

Stanczyk, F.Z., Namkung, P.C., Fritz, M.A., Novy, M.J., and Petra, P.H. (1986). The influence f sex steroid-binding protein on the metabolic clearance rate of testosterone. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 555-563. John Libbey Eurotext, London.

Strel'chyonok, O.A., Avvakumov, G.V., and Survilo, L.I. (1984). A recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium plasma membranes. *Biochim. Biophys. Acta* **802**, 459-466.

Thomas, P. (2000). Nuclear and membrane steroid receptors and their functions in teleost gonads. *In* "Reproductive Physiology of Fish 1999" (B. Norberg, O.S. Kjesbu, G.L.

Taranger, E. Anderson, and S.O. Stefansson, Eds.), pp. 149-156. John Grieg A/S, Bergen, Norway.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

Westphal, U. (1986). "Steroid-Protein Interactions II", 592p. Monographs on Endocrinology 27, Springer-Verlag, New York.

Wingfield, J.C. (1980). Sex steroid-binding proteins in vertebrate blood. *In* "Hormones, Adaptation and Evolution" (S. Ishii, T. Hirano, and M. Wada, Eds.), pp. 135-144. Springer-Verlag, Berlin.

2. Assay Development

2. Assay Development

2.1 Introduction

Methods which have been used to measure sex steroid binding protein (SBP) include binding assays which measure active SBP (such as equilibrium dialysis, adsorption methods such as dextran-coated charcoal (DCC), gel filtration and ammonium sulfate precipitation), and immunoassays which also measure inactive SBP (reviewed in Westphal, 1986). While it is generally recognised that equilibrium dialysis is the best method for determining active SBP in that there is the least disturbance of the equilibrium (Bennett, 1978), the time-consuming nature of this procedure can be limiting. A common alternative is to use DCC to separate bound and free steroid for assay of SBP (eg. Hryb *et al.*, 1986; Pottinger, 1988; Chang and Lee, 1992; Paolucci and Di Fiore, 1994). By this method, SBP is measured in a simpler manner than for example, equilibrium dialysis, allowing determination of SBP binding characteristics in laboratories where receptor characteristics are not routinely measured. The primary objection to use of DCC to separate bound and free steroid is that the addition of DCC to the assay disturbs the equilibrium of the reaction. However, this problem can be minimised by choice of an appropriately short incubation time with DCC (Clark and Peck, 1979). In the present study, a charcoal adsorption binding assay was investigated and optimised for measurement of SBP in rainbow trout *Oncorhynchus mykiss*, black bream *Acanthopagrus butcheri*, snapper *Pagrus auratus* and greenback flounder *Rhombosolea tapirina*. There is also the option of either hot or cold saturation protocols. In a hot saturation assay, the sample is incubated with increasing concentrations of radiolabelled ligand and a 100-fold excess of cold competitor. The advantages of this protocol are that specific binding can be measured at each radioligand concentration (McPherson, 1983). However, the disadvantage is that very high concentrations of radioligand may be necessary to enable saturation of the binding protein. In a cold saturation assay, the sample is incubated with a constant concentration of radioligand, and increasing concentrations of cold competitor, equivalent to addition of higher concentrations of radioligand of progressively lower specific activity (McPherson 1983). Non-specific binding is measured only at the first concentration of radioligand (McPherson, 1983). Provided there is sufficient binding at sub-k_D concentrations, a cold saturation assay allows use of much higher effective ligand concentrations.

Dilution of plasma prior to assay reduces the labelled and competing steroid concentrations required for saturation of the SBP. Dilution also helps meet the requirement that the smallest amount of binder be used such that the effects of excess binder on affinity estimations are minimised (Kenakin, 1993). The choice of dilution is between the minimum amount of binder able to be assayed consistently (Kenakin, 1993), and the maximum separation between specific and non-specific binding (Heyns, 1986). The plasma dilutions used for assays in the literature vary widely from a 10-fold dilution used for trout plasma (Pottinger and Pickering, 1990; Pottinger, 1988) to 200-fold dilutions used for spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1994) and goldfish *Carassius auratus* (Van Der Kraak and Biddiscombe, 1999) plasma. During optimisation of the assay for rainbow trout, black bream, greenback flounder and snapper in the present study, the effect of plasma dilution was investigated.

Ideally assays to determine differences between or within species should be performed using the same batch of buffer, labelled steroid and competitor, however, this is not always possible. Nor is it always possible to assay samples immediately after collection. Instead it is often necessary to store plasma until all samples are collected, and sufficient time is available to assay all related samples near or at the same time. The effect of storage of plasma on binding assay results is variable. Some authors have reported a substantial loss of binding activity with storage (Martin, 1975) whereas there appears to be a negligible effect in other studies (Idler and Freeman, 1973; Martin and Ozon, 1975; Lindstedt *et al.*, 1985). As plasma from companion studies by other workers in our laboratory was used for some aspects of the current study, it was necessary to investigate the effect of frozen storage and thawing of plasma on binding characteristics.

Endogenous steroids may also have an effect on the measurement of binding characteristics. This effect is generally expected to be minimal because of dilution of plasma, and high steroid concentrations added (Siiteri *et al.*, 1982; Pasmanik and Callard, 1986), however, stripping of endogenous steroids by DCC prior to assay is commonly used as a precaution (McPherson, *et al.*, 1988; Chang and Lee, 1992; Foucher *et al.*, 1992; Paolucci and Di Fiore, 1994). Therefore, various protocols using DCC to strip endogenous steroids from the plasma were examined.

In mammals, there is considerable competition between the high affinity, low capacity binding of E_2 and T to SBP, and low affinity, very high capacity binding of these steroids to albumin (Siiteri *et al.*, 1982). Hence albumin can have a considerable effect on the measured binding characteristics if it is not corrected for. An albumin-like protein is present in some fish (Maillou and Nimmo, 1993a, b), albeit at low concentrations in females (Maillou and Nimmo, 1993a). Binding of this albumin-like protein to steroids has not been investigated. Mammalian albumin is robust to heating at 60 °C , whereas SBP is denatured at this temperature (Westphal, 1971), enabling the contribution of albumin to be measured after heating of plasma. As teleost albumin-like proteins have some of the characteristics of mammalian albumin, measurement of binding remaining after heating of fish plasma to 60 °C was deemed to be a relatively easy way to determine if an albumin-like protein stable at higher temperatures was interfering with measurement of SBP binding characteristics.

These various aspects of the assay were investigated with the aim of producing an assay protocol that was robust and repeatable for comparative, and within species investigations.

2.2 Methods

Plasma Collection

Trout, flounder, bream and snapper were maintained or captured as described in Thomas *et al.* (1999), Barnett (1998), Haddy and Pankhurst (1998, 1999) and Cleary (1997) respectively. Blood samples were taken from female fish by caudal puncture with heparinised 22G needles. Blood samples were centrifuged (15000 g at 4 °C for trout and flounder, and 10000 g for bream and snapper) for 3 min and the plasma removed and stored at -20 °C.

Binding Assay Development

A protocol for a hot saturation charcoal adsorption binding assay (Van Der Kraak and Biddiscombe, 1999) was optimised for trout, flounder and bream. Several variations of the charcoal adsorption binding assay are reported in the literature. The length of time of

incubation for charcoal to adsorb free steroid, and the centrifuge time for separation of charcoal from the supernatant vary most widely. It has been suggested that a significant decrease in binding can occur with a relatively short increase in period of incubation with DCC (Laidley and Thomas, 1994). Incubation times used to measure SBP range from 30 s (Laidley and Thomas, 1997) to 15 min (Chang and Lee, 1992). Centrifuge times used to separate DCC and supernatant also vary from 5 min (Pottinger, 1986) to 15 min (Van Der Kraak and Biddiscombe, 1999). Therefore several charcoal incubation times (1-10 min) and centrifuge times (5, 10 and 15 min) were tested for trout, flounder and bream in the present study. Different assay incubation times (3, 6 and 18 h) were also tested for all species.

^{3}H-steroids ([2,4,6,7-^{3}H]estradiol (Amersham) and [2,4-^{3}H]estradiol (Sigma)) stored at -20 °C in ethanol were prepared for assay by evaporation under nitrogen and resuspended in 0.05 M phosphate buffer containing 0.1 % gelatine (pH 7.6) at the highest concentration required for the assay. Unlabelled steroid competitors were diluted in ethanol and then in phosphate buffer such that the final concentration of ethanol in each assay tube was 0.5 %. DCC-stripped plasma was diluted (200-fold for trout, 100-fold for bream and 50-fold for snapper and flounder) and incubated with a range of labelled E_2 concentrations both in the presence (3 tubes for non-specific binding) and absence (3 tubes for total binding) of 100-fold excess unlabelled E_2 as competitor. The ^{3}H-E_2 concentrations were originally 1, 2, 3.5, 5, 7.5, 10 and 20 nM for trout, bream and snapper, and 5, 7.5, 10, 15, 20, 30 and 40 nM for flounder. However, the range was increased to 0.05 - 20 nM (0.05, 0.1, 0.25, 0.5, 1, 2, 5, 7.5, 10 and 20 nM) for trout assays, to provide points with ^{3}H-E_2 concentrations below k_D. The low points here gave artefactual results (discussed later) so concentrations from 0.25 - 20 nM were used for all subsequent trout assays. Flounder assays were initially performed at a 20-fold dilution with a range of ^{3}H-E_2 concentrations from 5 - 40 nM, but this protocol was subsequently changed because the specific binding component in the plasma was not saturated with this range of ^{3}H-E_2 concentrations. For all other estimations of k_D and B_{max} for flounder, a cold saturation protocol was adopted where DCC-stripped, 50-fold diluted flounder plasma was incubated with 5 nM ^{3}H-E_2 and increasing concentrations (5, 50, 500, 2500 nM) of unlabelled E_2. Tubes were equilibrated for 30 min at 20 °C and overnight at 4 °C. The incubation was terminated by addition of 0.5 ml 10 mg.ml^{-1} charcoal coated with 0.05 % dextran. Tubes were vortexed, incubated at 4 °C

for 5 min and centrifuged at 3000 rpm and 4 °C for 10 min. The supernatant was decanted, 5 ml of Ecolite scintillation cocktail (ICN Biochemicals) added, and vials were shaken and counted using a standard tritium procedure. Specific binding was calculated by subtracting non-specific binding from total binding.

The Scatchard plots for the trout showed a 'hook' at low ^{3}H-E_2 concentrations. To determine whether this was generated by positive cooperativity, or was a common assay artefact resulting from the degradation of unliganded protein (Chamness and McGuire, 1975), diluted trout plasma from 5 fish that had been incubated with a low concentration of ^{3}H-E_2 (0.05 nM) overnight, was re-incubated with a high concentration of ^{3}H-E_2 (10 nM), and the counts from these tubes were compared to counts from tubes incubated with 10 nM (high) ^{3}H-E_2 for the entire incubation time.

The equilibrium dissociation constant (k_D), maximum number of binding sites (B_{max}), contribution of non-specific binding, and the number of binding components was determined using the computer programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980). EBDA provides estimates of k_D and B_{max} to use as starting estimates in LIGAND. For hot saturation experiments, estimates are calculated from Scatchard plots by linear regression of selected points. For competition studies, an iterative curve fitting program starts with estimates from the sigmoidal curve to provide starting values for use in LIGAND. In LIGAND, the parameter values are fed into a mathematical model of the ligand-binding system. Use of the mathematical model avoids biases associated with calculations based on simple approximations such as Scatchard plots. In the present study, the results are still presented as Scatchard plots, however, the points and resulting line from LIGAND are used. Using a mathematical model also allows the data to be analysed for more than one binding site if curvilinear Scatchard plots are obtained. Another advantage of LIGAND is the statistically objective measure of goodness of fit of the model to the experimental points. Several assumptions are made for analysis in LIGAND. The labelled and unlabelled ligands are assumed to be chemically identical, experiments are assumed to have reached equilibrium, and a constant percent error in the 'bound' measurements is also assumed.

Plasma Dilution

During optimisation of the assay, single point assays at dilutions from 10- to 200-fold were performed for each species to determine the effect of dilution on specific and non-specific binding. The effect of plasma dilution on binding protein saturation and values measured for k_D and B_{max} was also investigated. Assays were performed with plasma at dilutions of 50-, 100- and 200-fold for all 4 species . Additionally, dilution factors of 10- and 20-fold for snapper plasma, and 5- and 20-fold for flounder plasma were investigated as specific binding was greater than non-specific binding at high dilutions in these 2 species. The k_D and binding capacity were calculated according to Scatchard (1949).

Plasma Storage

The effect of storage of trout, bream and flounder plasma at -20 °C was investigated by comparing binding assays on the same plasma sample assayed before and after freezing. To determine whether plasma that had previously been thawed and refrozen could be used for assay, comparisons of binding affinity and capacity were made for plasma thawed once and assayed, then refrozen, thawed and assayed a second time. Fresh snapper plasma was not available in Tasmania. To investigate the effect of storage of trout and flounder plasma, assays were performed with labelled E_2 concentrations of 1 - 20 nM and 5 - 40 nM respectively. Assays for bream were as described above.

Stripping Endogenous Steroids

An experiment was performed to test the effect of stripping endogenous steroids from the plasma of trout, flounder and bream prior to assay. Snapper plasma was not available at the time of this experiment. Pools of whole plasma were incubated with dextran-coated charcoal (DCC; 0, 5, 10 or 20 $mg.ml^{-1}$ charcoal with 0, 0.5, 1 or 2 $mg.ml^{-1}$ dextran respectively), for 5, 10 or 20 min. The tubes were vortexed after the DCC was added, and again after 10 min of the 20 min incubation time. The tubes were then centrifuged at 2060 g and 4 °C for 10 min. The supernatant was collected and stored at -20 °C. Levels of E_2 and T in the stripped plasma were measured by radioimmunoassay (RIA) as described by Pankhurst and Conroy (1987). Estimates of k_D and B_{max} were calculated according to the method of Scatchard (1949) from assays performed with labelled E_2 concentrations of

1 - 20 nM for trout and bream, and 5 - 40 nM for flounder. In a second experiment, plasma that had been stripped for 10 min with 10 $mg.ml^{-1}$ DCC was stripped a second time with 10 $mg.ml^{-1}$ DCC for 10 min and centrifuged for 10 min. The steroid levels of the decanted supernatant were measured by RIA. In a third experiment which also included snapper, whole plasma was incubated with 20 $mg.ml^{-1}$ DCC for 10, 40 or 60 min with 10 s vortexing every 2 min. Tubes were then centrifuged at 2060 g and 4 °C for 5 min. The supernatant was collected and assayed as above.

Low Affinity Binding

Pools of plasma from trout, flounder, bream and snapper were assayed after heating at 60 °C for 1 h (to denature SBP; Westphal, 1971) and equilibration at 12 °C for 1 h, to determine the extent of interference by binding of steroid to a low affinity binder such as albumin or an albumin-like protein. Plasma from the same pool was assayed without heating as a control. To investigate low affinity binding to trout and bream plasma, assays were performed with labelled E_2 concentrations of 1 - 20 nM. A range of ^3H-E_2 concentration from 5 - 40 nM, and a plasma dilution of 20-fold was used for assays of flounder plasma.

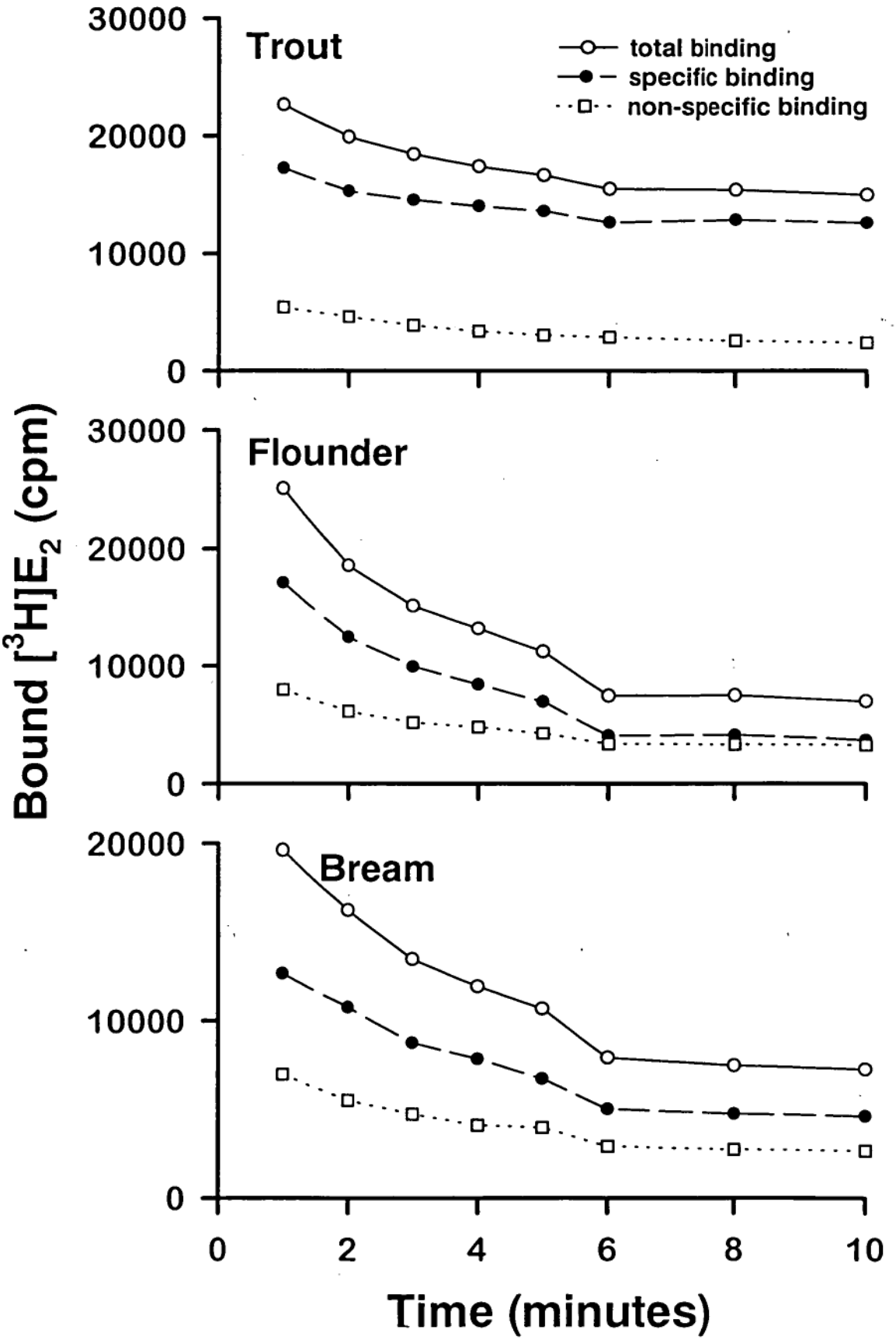
2.3 Results

Binding Assay Development

Increasing the time of incubation with DCC for separation of bound and free steroid resulted in a decrease in binding over the first 6 min of the incubation (Fig. 2.1). Binding remained stable from 6 to 10 min. Binding in trout plasma decreased by about 25 % during the first 6 min, whereas binding in the flounder and bream plasma decreased by more than 60 %. A DCC incubation time of 5 min was used for subsequent assays, as specific binding always remained higher than non-specific binding, and assay size with available equipment was greatly limited with any shorter incubation times.

The three centrifuge times (5, 10 and 15 min), tested for the separation of DCC-bound steroid from SBP-bound steroid did not affect binding at a labelled steroid concentration of

Figure 2.1: Decrease in binding of steroid with increasing charcoal incubation times for trout, flounder and bream. 10 nM ^{3}H-E_2 and 1 mM unlabelled E_2 competitor were used.


Trout
total binding
specific binding
non-specific binding
Flounder
Bream
30000
20000
10000
0
Bound [^{3}H]E_2 (cpm)
0
2
4
6
8
10
Time (minutes)

10 nM in trout, flounder and bream (data not shown) and 10 min was used for all subsequent assays. There were no obvious differences between assays incubated for 3, 6 or 18 h (data not shown) so assays were incubated overnight (about 18 h) for logistical reasons.

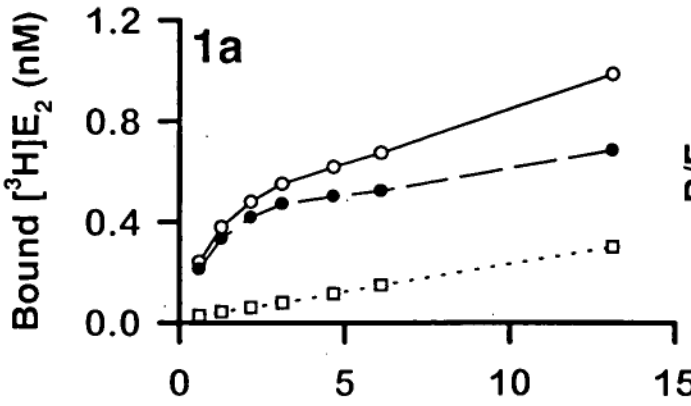
The initial range of ^{3}H-E_2 concentrations for trout assays (1 - 20 nM) did not provide points above and below k_D (Fig. 2.2 (1a, b)), so subsequent assays were performed using a range of ^{3}H-E_2 concentrations from 0.05 - 20 nM. A 'hook' was apparent at low ^{3}H-E_2 concentrations used in these trout assays comprising 2 points with lower than expected bound/free values at low bound E_2 concentrations (Fig. 2.2 (2a, b)). Specific binding was lower in plasma that had been incubated with low and then high concentrations of ^{3}H-E_2 compared to specific binding remaining after incubation only with high ^{3}H-E_2 suggesting degradation of unliganded SBP at low ligand concentrations. This difference was significant using a paired t-test (mean difference = 11.1 %, $p = 0.0126$). The hook points were removed from subsequent assays (Fig. 2.2 (3a, b)).

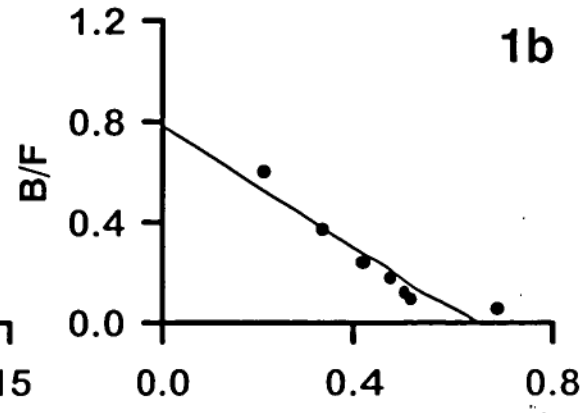
A number of problems were experienced with the assay for SBP in flounder. Plasma was initially assayed with a hot saturation protocol with labelled E_2 concentrations ranging from 5 - 40 nM. However, the points on the Scatchard plots of these assays tended to be widely scattered around the fitted line, and the binding component in the plasma was not saturated (Fig. 2.3 (1a, b)). Investigation of the effect of low affinity binding (described below) and use of serum rather than plasma (data not shown) did not improve the assay for flounder. The assay was subsequently changed to a cold saturation protocol (the constant amount of labelled steroid and increasing concentrations of unlabelled steroid effectively providing an assay with increasing concentrations of labelled steroid of decreasing specific activity). Binding with this protocol reached saturation (Fig. 2.3 (2a, b)). A single hot saturation assay with ^{3}H-E_2 concentrations ranging from 5 - 160 nM was performed to confirm k_D and B_{max} estimates from the cold saturation assays (Fig. 2.3 (3a, b)).

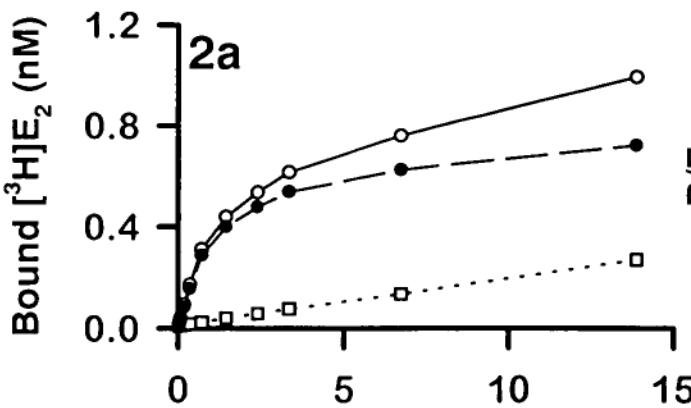
Plasma Dilution

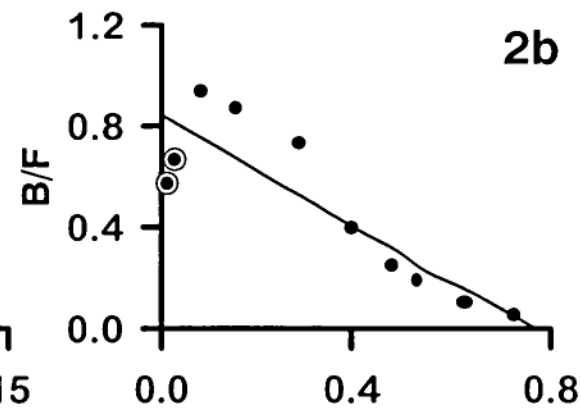
A 50-fold dilution of trout plasma (2 % plasma) gave the best separation of specific and non-specific binding (Fig. 2.4), but when a Scatchard assay was performed at this dilution

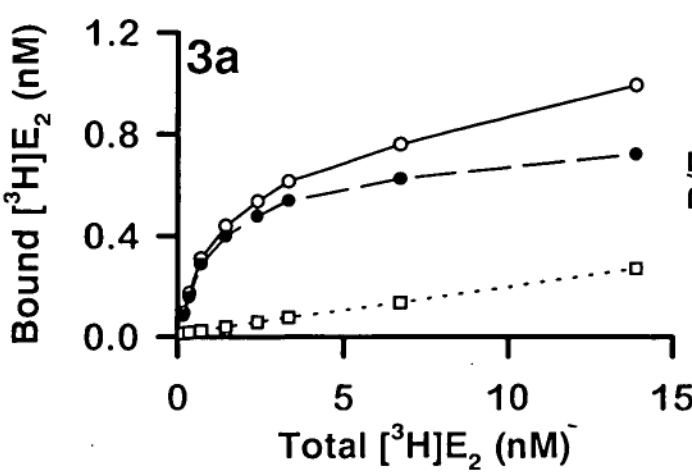
Figure 2.2: Saturation plots (a) showing total (hollow circle), specific (filled circle) and non-specific (hollow square) binding, and Scatchard plots (b) from assays of trout plasma. 1. Assay with ^{3}H-E_2 concentrations ranging from 1 - 20 nM. 2. Assay with ^{3}H-E_2 concentrations ranging from 0.05 - 20 nM. The 2 points comprising the 'hook' are circled. 3. Assay with ^{3}H-E_2 concentrations ranging from 0.2 - 20 nM.


1a
Bound [^{3}H]E$_2$ (nM)
1.2
0.8
0.4
0.0
0
5
10
15


1b
B/F
1.2
0.8
0.4
0.0
0.0
0.4
0.8


2a
Bound [^{3}H]E$_2$ (nM)
1.2
0.8
0.4
0.0
0
5
10
15


2b
B/F
1.2
0.8
0.4
0.0
0.0
0.4
0.8


3a
Bound [^{3}H]E$_2$ (nM)
1.2
0.8
0.4
0.0
0
5
10
15
Total [^{3}H]E$_2$ (nM)

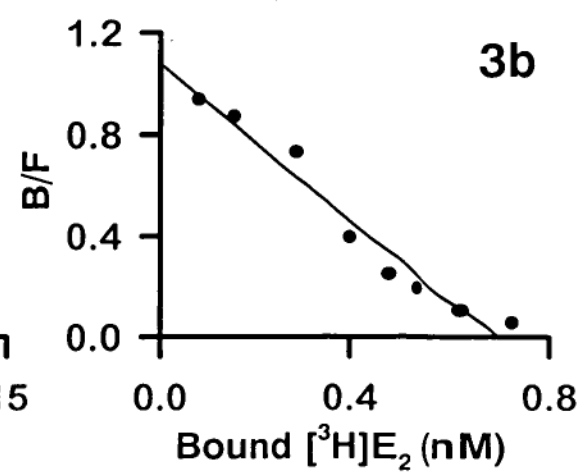
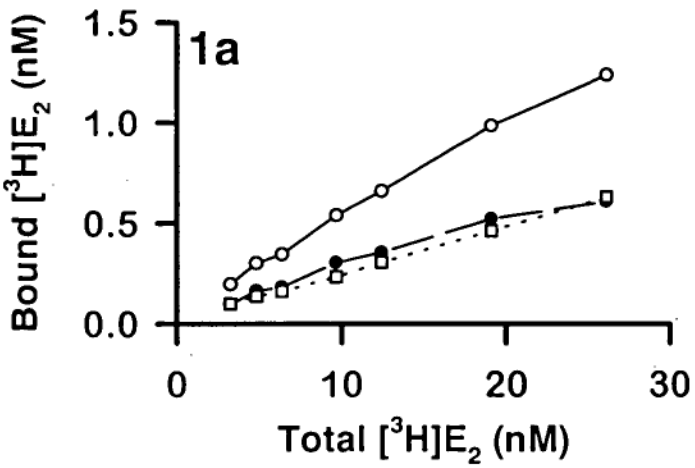
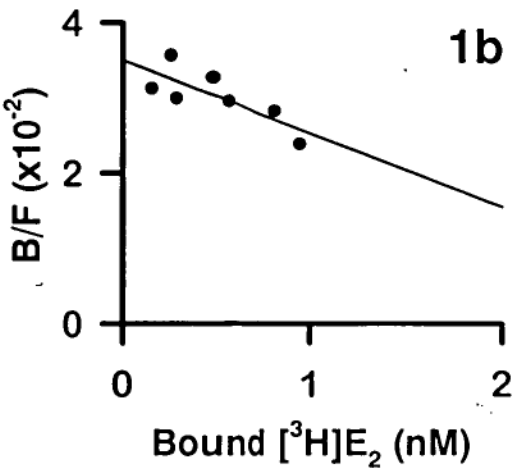
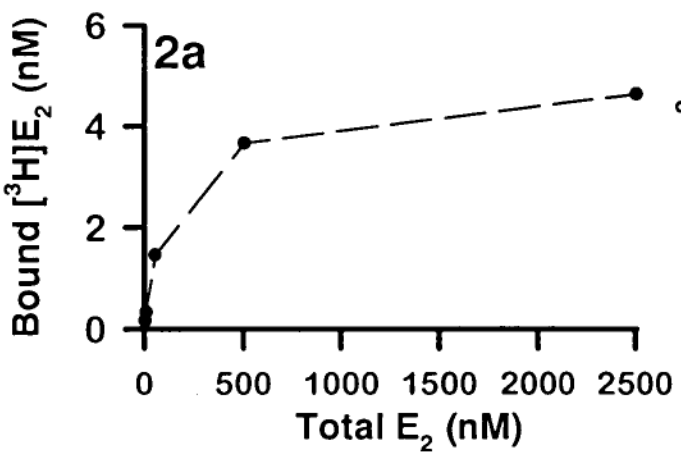
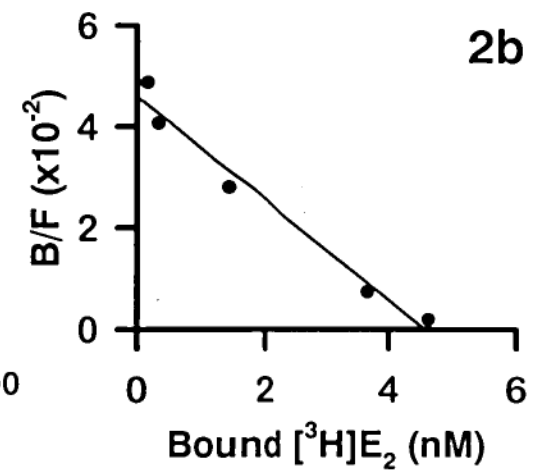

3b
B/F
1.2
0.8
0.4
0.0
0.0
0.4
0.8
Bound [^{3}H]E$_2$ (nM)

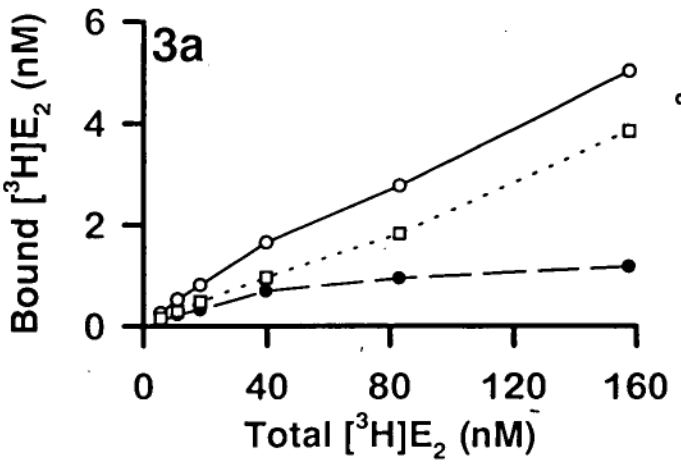
Figure 2.3: Saturation plots (a) showing total (hollow circle), specific (filled circle) and non-specific (hollow square) binding, and Scatchard plots (b) from assays of flounder plasma. 1. Hot saturation assay with ^{3}H-E_2 concentrations ranging from 5 - 40 nM. 2. Representative cold saturation assay. The saturation plot shows only specific binding as non-specific binding was measured at only a single ^{3}H-E_2 concentration. 3. Hot saturation assay with ^{3}H-E_2 concentrations from 5 - 160 nM.


1a
Bound [^{3}H]E$_2$ (nM)
1.5
1.0
0.5
0.0
0
10
20
30
Total [^{3}H]E$_2$ (nM)


1b
B/F (x10^{-2})
4
2
0
0
1
2
Bound [^{3}H]E$_2$ (nM)


2a
Bound [^{3}H]E$_2$ (nM)
6
4
2
0
0
500
1000
1500
2000
2500
Total E$_2$ (nM)


2b
B/F (x10^{-2})
6
4
2
0
0
2
4
6
Bound [^{3}H]E$_2$ (nM)


3a
Bound [^{3}H]E$_2$ (nM)
6
4
2
0
0
40
80
120
160
Total [^{3}H]E$_2$ (nM)

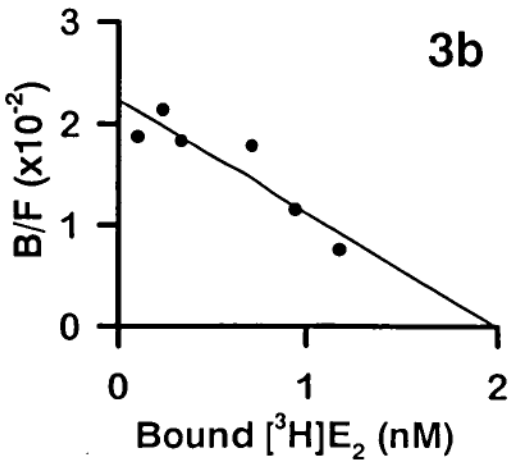
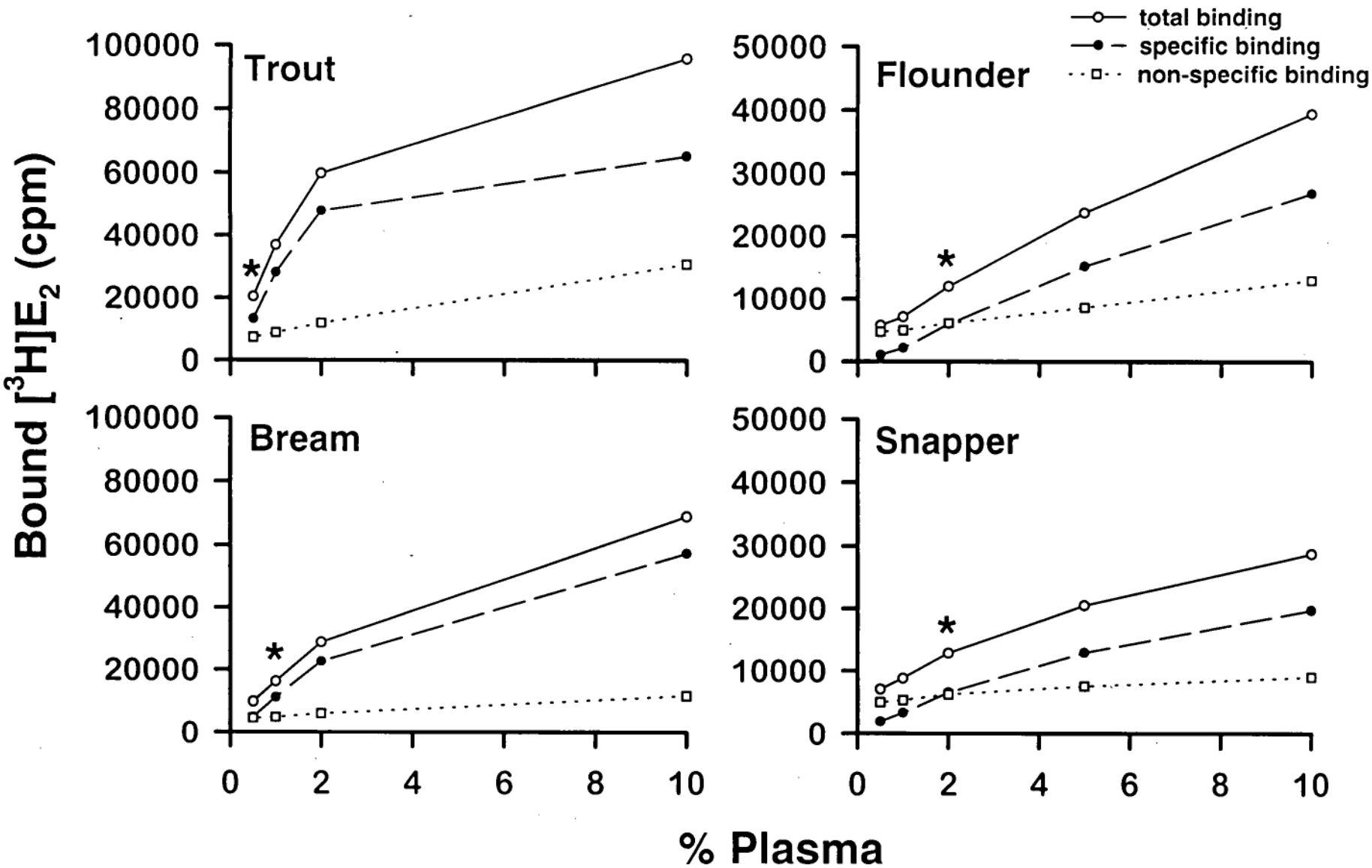

3b
B/F (x10^{-2})
3
2
1
0
0
1
2
Bound [^{3}H]E$_2$ (nM)

Figure 2.4: Change in total, specific and non-specific binding of steroid with plasma concentration for trout, flounder, bream and snapper. 10 nM ^{3}H-E_2 and 1 mM unlabelled E_2 competitor were used. Plasma dilutions used for subsequent assays are marked with *.


Trout
Flounder
Bream
Snapper
total binding
specific binding
non-specific binding
Bound [^{3}H]E$_2$ (cpm)
% Plasma
100000
80000
60000
40000
20000
0
50000
40000
30000
20000
10000
0
2
4
6
8
10
*

(data not shown), the binding protein was not saturated at the highest concentration of label. A 200-fold dilution of plasma allowed saturation of the binding protein within the range of label concentrations used, and was used for all subsequent trout binding assays, although there was less separation of specific and non-specific binding at this dilution. Specific binding in flounder plasma decreased steadily with increasing plasma dilution and was just below the level of non-specific binding at 50-fold dilution (Fig. 2.4). A 20-fold plasma dilution was used initially, however, in subsequent assays, flounder plasma was diluted 50-fold. A 10-fold dilution of bream plasma gave the best separation of specific and non-specific binding (Fig. 2.4), but a 100-fold plasma dilution gave the best Scatchard curve and was used for all subsequent bream binding assays. The specific binding curve for snapper showed a similar pattern to that for flounder, with non-specific binding higher than specific binding at dilutions greater than 50-fold (Fig. 2.4). Assays at dilutions other than 50-fold, however, were not as satisfactory as at 50-fold (Fig. 2.5), therefore a 50-fold dilution was used in all assays. At this plasma concentration, non-specific binding was generally around 50 % of the total binding.

Plasma Storage

There were no obvious trends within the species of changing affinity or capacity with plasma storage time. Lower estimates for k_D and B_{max} were obtained from a sample of trout plasma that had been stored for 6 months compared to assays of the same plasma at all other times, however, the 95 % confidence limits on these estimates all overlapped (Table 2.1). The B_{max} of bream plasma stored frozen for up to 12 months did not differ from that of plasma assayed fresh (Table 2.2). The k_D of plasma stored for up to 6 months and thawed a single time for assay did not differ from the k_D calculated from fresh plasma. However, the k_D of plasma that had been frozen for 12 months was higher than the k_D of plasma that had been thawed twice during 1 month of storage. The binding characteristics of flounder plasma that had been stored for up to 1 month did not differ from those of fresh plasma (Table 2.3).

Figure 2.5: Scatchard plots from assays of snapper plasma at dilutions of 20-, 50-, 100- and 200-fold.

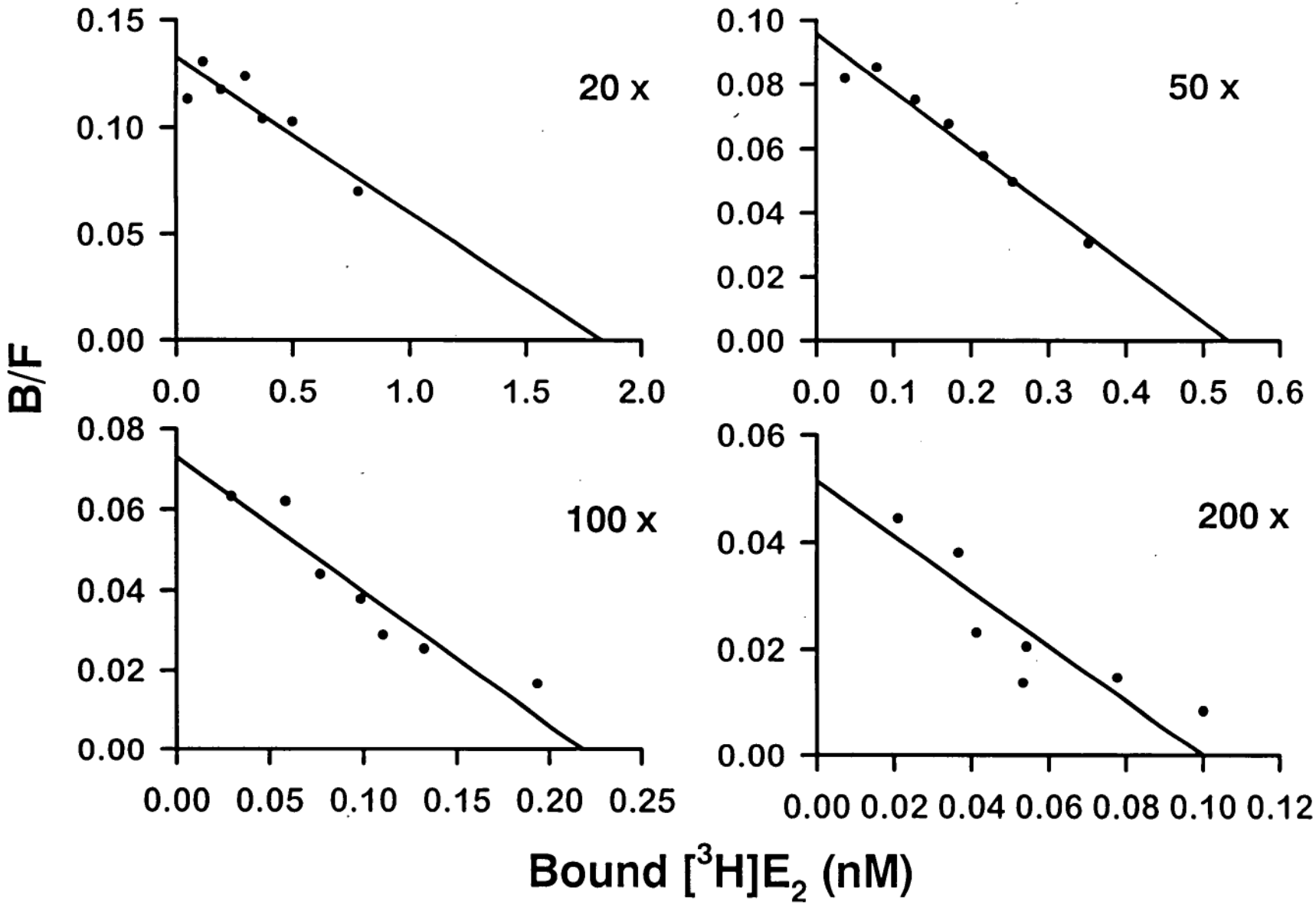

B/F
20 x
50 x
100 x
200 x
0.15
0.10
0.05
0.00
0.0 0.5 1.0 1.5 2.0
0.10
0.08
0.06
0.04
0.02
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6
0.08
0.06
0.04
0.02
0.00
0.00 0.05 0.10 0.15 0.20 0.25
0.06
0.04
0.02
0.00
0.00 0.02 0.04 0.06 0.08 0.10 0.12
Bound [3H]E2 (nM)

Table 2.1: Estimates of binding affinity and capacity, and their associated 95 % confidence limits for a trout plasma sample assayed fresh and after frozen storage for various times.

	k_D (nM)	(95 % CL)	B_{max} (nM)	(95 % CL)
fresh	0.58	(0.40, 0.85)	120	(108, 132)
frozen 1 week	0.50	(0.28, 0.90)	113	(98, 130)
frozen 1 month	0.43	(0.27, 0.68)	116	(105, 128)
frozen 1 month, thawed twice	0.47	(0.30, 0.74)	119	(107, 132)
frozen 6 months	0.34	(0.14, 0.80)	95	(78, 112)

Table 2.2: Estimates of binding affinity and capacity, and their associated 95 % confidence limits for a bream plasma sample that was assayed fresh and after frozen storage for various times. Estimates of k_D that are significantly different (non-overlapping confidence limits from LIGAND) are shown by different superscripts.

	k_D (nM)	(95 % CL)	B_{max} (nM)	(95 % CL)
fresh	2.82 ^{ab}	(2.25, 3.54)	48.1	(42.1, 54.9)
frozen 1 week	2.48 ^{ab}	(1.60, 3.85)	37.1	(29.3, 47.0)
frozen 1 month	2.55 ^{ab}	(1.86, 3.50)	37.4	(31.6, 44.2)
frozen 1 month, thawed twice	2.19 ^{a}	(1.63, 2.95)	32.2	(27.8, 37.4)
frozen 6 months	3.22 ^{ab}	(2.64, 3.92)	34.7	(31.0, 38.9)
frozen 12 months	5.71 ^{b}	(3.44, 9.46)	43.6	(31.2, 61.0)

Table 2.3: Estimates of binding affinity and capacity, and their associated 95 % confidence limits for a flounder plasma sample assayed fresh and after frozen storage for various times.

	k_D (nM)	(95 % CL)	B_{max} (nM)	(95 % CL)
fresh	30.2	(17.2, 52.9)	437	(282, 676)
frozen 1 week	46.1	(23.5, 90.7)	428	(246, 746)
frozen 1 month	40.8	(28.2, 59.2)	588	(432, 800)

Stripping Endogenous Steroids

The binding capacity of unstripped plasma compared to plasma stripped with 20 $mg.ml^{-1}$ DCC for 20 min was 4-15 % lower in trout and 30-47 % lower in bream (data not shown). Due to lack of saturation of the binding site with the initial assay protocol for flounder, values for B_{max} could not be calculated from the assays performed with DCC-stripped flounder plasma, and these assays were not carried out with snapper plasma. Steroid levels in stripped plasma were lower than in unstripped plasma, but DCC stripping did not appear to remove all endogenous steroid. Neither did the level of stripping change appreciably with increasing DCC concentration, or increasing incubation time (data for trout are shown in Table 2.4). A second stripping did not further reduce the concentration of E_2 in the plasma in any species. The concentration of T was reduced in flounder and bream plasma (data not shown) but not in trout plasma after double-stripping (Table 2.4). Longer incubation times with the highest charcoal concentration did not reduce the steroid concentration remaining in trout plasma below what had been achieved with the lowest DCC concentration and the shortest time (Table 2.5). Initial steroid levels in bream, flounder and snapper plasma were lower and the results were less clear. Increased incubation time resulted in progressively lower plasma levels of T but not E_2 in bream and flounder, whereas E_2 was reduced to below the assay detection limit at all stripping times and T was below the detection limit in unstripped plasma for snapper (Table 2.5). The highest DCC concentration of 20 $mg.ml^{-1}$ was chosen, and for logistical reasons an intermediate incubation time of 20 min was used for subsequent assays.

Low Affinity Binding

Low affinity binding (as determined by measurement of binding after heat denaturation of SBP) was not saturable at the labelled steroid concentrations used for SBP assays (Fig. 2.6). Low affinity binding was a smaller component of total binding than non-specific binding for each of these species. However, at high concentrations of labelled steroid, non-specific binding was approaching, or higher than specific binding in trout, bream and snapper, and low affinity binding was close to the level of non-specific binding in trout and snapper. After correction for low affinity binding, specific binding to SBP was clearly saturated at the steroid concentrations used in assays for trout, bream, and snapper, but not

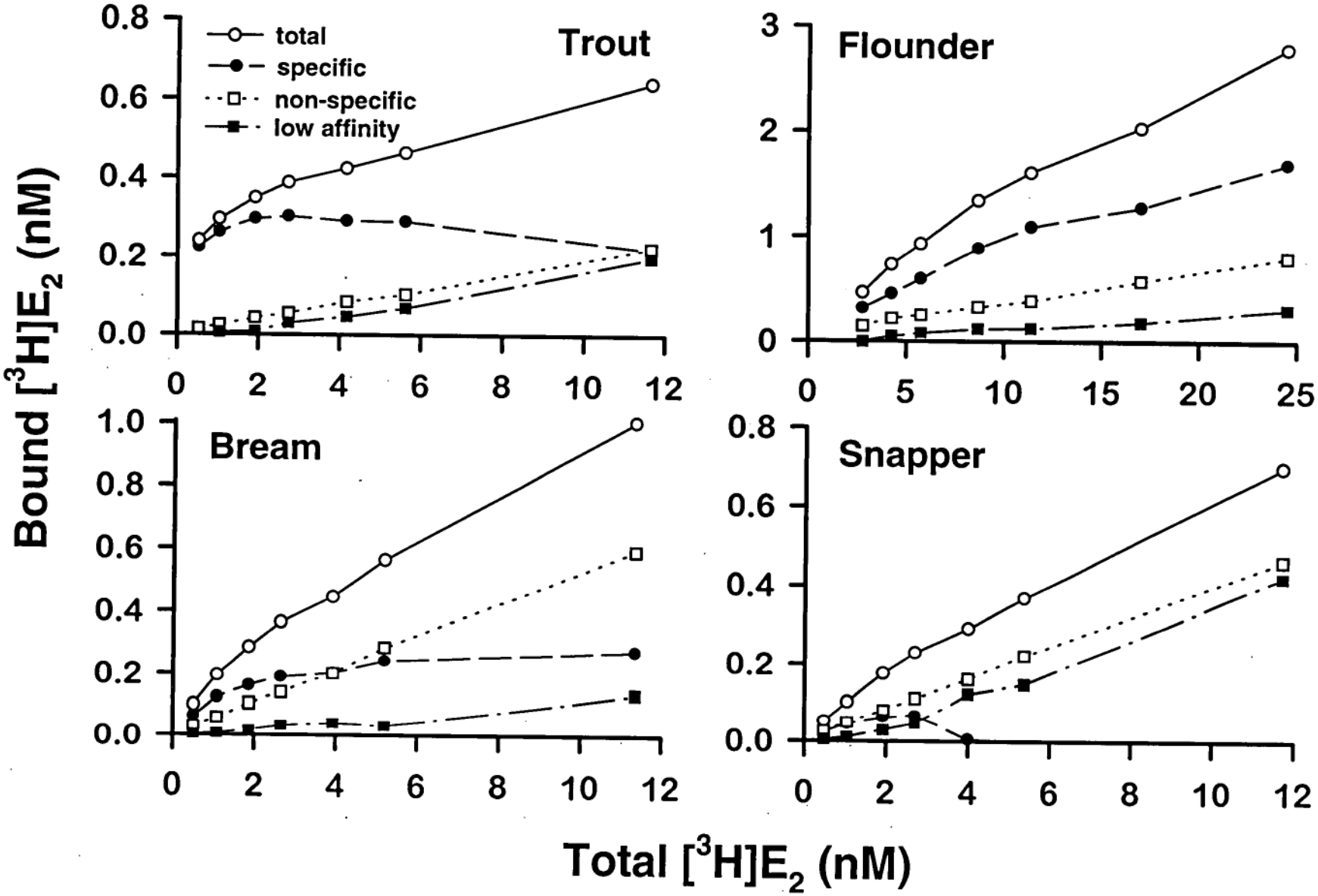
Table 2.4: Concentrations of E_2 and T in trout plasma, prior to, and following stripping with dextran-coated charcoal at three concentrations for three incubation times, and after double-stripping with 10 $mg.ml^{-1}$ DCC. Unstripped values were 20.1 and 36.9 $ng.ml^{-1}$ for E_2 and T respectively.

Stripping time	E_2 ($ng.ml^{-1}$)		
	5 $mg.ml^{-1}$ DCC	10 $mg.ml^{-1}$ DCC	20 $mg.ml^{-1}$ DCC
5 min	6.4	7.6	7.7
10 min	7.0	6.4	8.3
20 min	6.1	7.5	7.8
2x 10 min		10.7	
		T ($ng.ml^{-1}$)	
5 min	13.6	16.4	18.4
10 min	-	16.3	20.6
20 min	15.2	-	19.1
2x 10 min		15.0	

Table 2.5: Plasma concentrations of E_2 and T for trout, flounder bream and snapper, prior to and following, stripping with 20 $mg.ml^{-1}$ DCC for between 10 and 60 min.

	Time (min)	E_2 ($ng.ml^{-1}$)	T ($ng.ml^{-1}$)
trout	unstripped	20.1	36.9
	10	7.5	26.0
	20	9.2	24.8
	60	8.3	23.8
flounder	unstripped	0.9	6.9
	10	<0.3	3.3
	20	<0.3	2.5
	60	1.4	1.5
bream	unstripped	1.0	1.7
	10	1.5	1.4
	20	1.1	<0.3
	60	0.9	<0.3
snapper	unstripped	5.8	<0.3
	10	<0.3	<0.3
	20	<0.3	<0.3
	60	<0.3	<0.3

Figure 2.6: Composite saturation curves of heat-treated and non-heated plasma from trout, flounder, bream and snapper. Total and non-specific binding were from unheated plasma. Low affinity binding was specific binding remaining after heat-treatment of plasma. Specific binding was specific binding from untreated plasma with low affinity binding subtracted.


total
specific
non-specific
low affinity
Trout
Flounder
Bream
Snapper
Bound [3H]E2 (nM)
Total [3H]E2 (nM)

for flounder (Fig. 2.6). Specific binding in snapper plasma appeared to decrease as ^{3}H-E_2 increased above 3 nM. However, this appeared to result from high levels of non-specific and low affinity binding at high concentrations of ^{3}H-E_2. Small differences in measurement of binding between the assay with heated snapper plasma and with non-heated plasma combined to show the apparent, but probably not real, reduction in specific binding. Plasma was not routinely heated to correct for low affinity binding.

2.4 Discussion

Binding Assay Development

Optimisation of the charcoal adsorption assay protocol resulted in consistent Scatchard plots for plasma binding from trout, bream and snapper (see Chapter 3, this volume). The hook on the trout Scatchard plots resulting from points at low ^{3}H-E_2 concentrations appeared to result from degradation of unliganded SBP at low ligand concentrations rather than positive cooperativity. In view of this fact, the 2 lowest points were removed from trout assays so the lowest ^{3}H-E_2 concentration became 0.25 nM rather than 0.05 nM. This point was still lower than k_D and Hill plots of subsequent assays still had points either side of 0. Hill coefficients for all trout assays ranged from 0.63 to 1.02 giving no evidence of cooperative binding, and supporting the view that the 'hook' was an artefact. Binding in the flounder assay was variable and not saturable at the ligand concentrations and plasma dilution initially used in the present study. Neither was the specific binding saturable using more dilute plasma (Hobby and Pankhurst, 1997). Changing the protocol to a cold saturation assay allowed saturation of the protein. Estimates of k_D and B_{max} from the cold saturation assays (k_D = 85 nM and B_{max} = 164 nM; see Chapter 3, this volume) were confirmed by performing a single hot saturation assay with higher labelled E_2 concentrations. The estimates of binding affinity (85 nM) and capacity (96 nM) from this assay agreed fairly well with values from the cold saturation assays, and the cold saturation protocol was adopted for the remaining flounder assays. However, there is a possibility of overestimation of B_{max} with the cold saturation method. The hot saturation assay was not performed repeatedly due the high ^{3}H-E_2 concentration required. Regardless of whether flounder plasma was assayed with the hot or cold saturation protocol, the assays were not as reproducible as those with the other fish species. This may result from the lower

binding affinity and very rapid dissociation rate compared to trout, bream and snapper (Chapter 3, this volume).

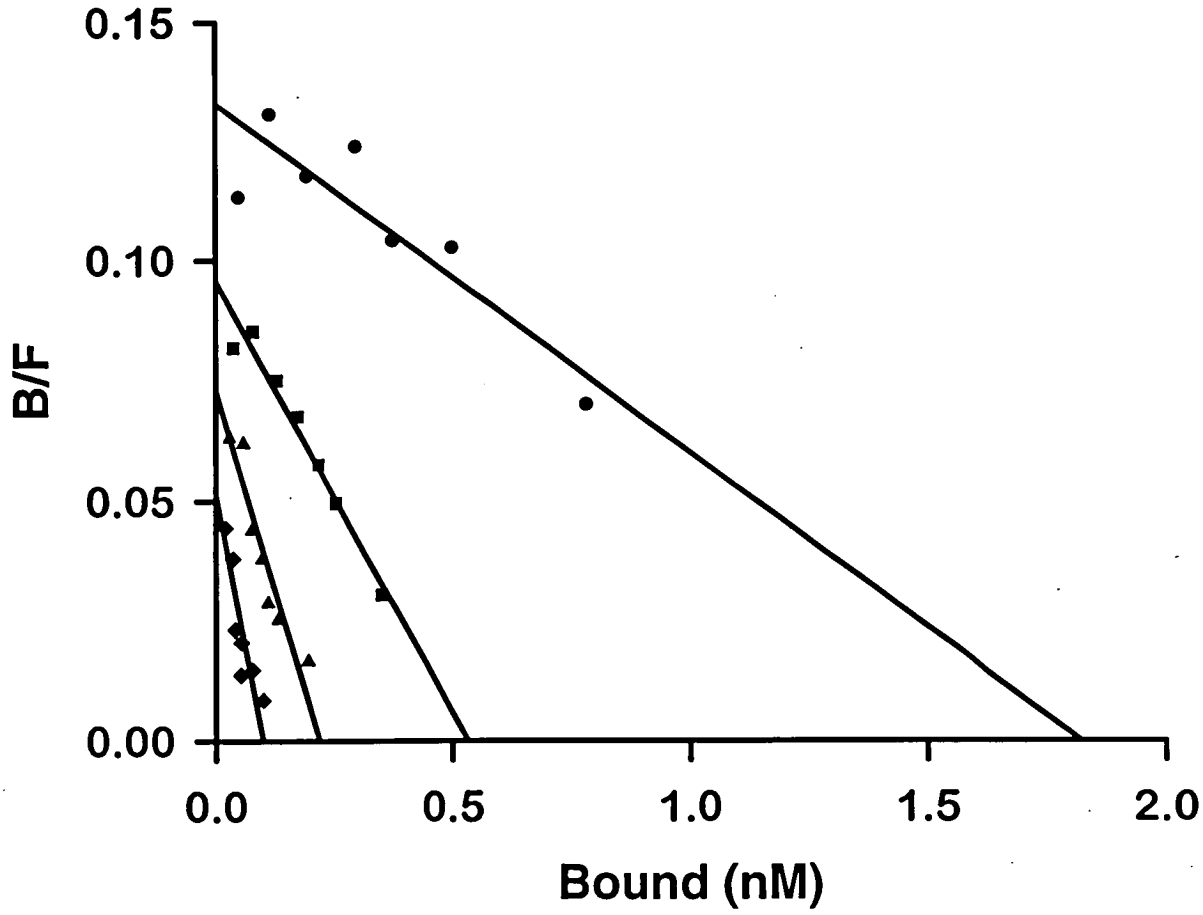
Plasma Dilution

The plasma dilutions that gave the best Scatchard plots varied between fish species. A 200-fold dilution was optimal for trout plasma, whereas a 100-fold dilution of plasma gave the tidiest assays with bream plasma, and a 50-fold dilution was best with snapper and flounder plasma. Measuring binding protein characteristics at different dilutions presents problems for comparative studies because estimates of k_D increase with dilution for the same species (Fig. 2.7), and this did not appear to be an effect of negative cooperativity (Hill plot slope ~1 for bream, snapper and trout). Other investigators have failed to find an explanation for this effect (Siiteri *et al.*, 1982), but it may be related to the non-linear binding of steroids to SBP and albumin in humans, where the amount of steroid bound is substantially influenced by the concentrations of the proteins (Zeginiadou *et al.*, 1997). Alternatively, the assumption that the quantity of binding protein does not substantially alter the concentration of free ligand (Kenakin, 1993) may not be correct in this case. This raises questions as to whether we can compare affinity values between species, when the assays were performed at different dilutions of plasma. Assays of trout and bream plasma at 50-fold dilution were not as satisfactory as assays at the chosen dilutions, but values for k_D and the binding capacity could be extracted from the respective Scatchard plots to be compared with snapper and flounder assays at 50-fold dilution. The order of binding affinities remained the same as reported elsewhere (ie. trout > bream > snapper >> flounder; Chapter 3, this volume), however, the differences between the k_D values for the different species were not as large from assays at the same dilution.

Plasma Storage

Storage of plasma at -20 ^{0}C did not affect either k_D or B_{max} in a consistent manner. There was a hint of a reduction in B_{max} in trout plasma after frozen storage for 6 months. However, B_{max} measured in a sample of bream plasma stored for 12 months was almost identical to that measured from the same plasma when fresh, and flounder plasma if anything showed a slight increase in B_{max}. There was also a suggestion of lower k_D in trout

Figure 2.7: Scatchard plots for a sample of snapper plasma diluted 20-, 50-, 100- and 200-fold for assay. Estimates of k_D and B_{max} were calculated in LIGAND. Parameters with non-overlapping confidence limits are shown by different superscripts.



Symbol	Dilution factor	k_D	B_{max} (corrected for dilution)
●	20	13.76 [a]	36.5 [a]
■	50	5.57 [b]	26.6 [a]
▲	100	2.99 [bc]	21.8 [a]
◆	200	1.95 [c]	20.0 [a]

plasma that had been stored for 6 months, however, once again the data from stored bream and flounder plasma were inconsistent with this, suggesting a possible increase in k_D with extended frozen storage. These measurements were only from a single sample at each time, so these possible differences could well result from between assay variation. These results suggesting that SBP in trout, flounder and bream is relatively robust to frozen storage are in agreement with reports that human SBP shows reasonable stability in serum samples, regardless of repeated freezing and thawing (Lindstedt *et al.*, 1985). The activity of amphibian and salmon SBP also remains constant with storage at -17 to -18 °C (Martin and Ozon, 1975, Idler and Freeman, 1973), and SBP from spotted seatrout remains active after storage for several weeks at 4 °C (Laidley and Thomas, 1994). The apparent stability of SBP to frozen storage reported here is in contrast to a study on dogfish *Scyliorhinus canicula* SBP where a substantial reduction in binding capacity was found after storage at -18 °C for 4 weeks (Martin, 1975). Plasma from cod and haddock *Melanogrammus aeglefinus* also showed a loss of binding capacity when stored at -17 °C, but not following storage at -40 °C (Idler and Freeman, 1973). Due to the requirement for use of plasma from other studies, assays for the present study could not always be performed on plasma that had not been thawed previously. However, plasma that had not been stored for long periods, or thawed since it was frozen was used wherever possible.

Stripping Endogenous Steroids

The effect of endogenous steroids on the measured binding capacity in the absence of any procedure to remove them, is generally assumed to be negligible because their concentration after dilution of plasma is low relative to the added steroid (Siiteri *et al.*, 1982; Pasmanik and Callard, 1986). However, the apparent underestimation of B_{max} (~ 30 - 47 % and ~ 4 - 15 % lower for unstripped bream and trout plasma respectively) suggests the effect of endogenous steroids may not be substantially reduced by dilution. Stripping of steroids from SBP by DCC depends on the adsorption constant of DCC for steroid and its high capacity, versus the association constant of SBP. It is likely that free steroid is adsorbed first by DCC, and then steroid dissociating from SBP to maintain the equilibrium is adsorbed. Contrary to expectations, incubation with DCC for up to 1 h did not consistently remove all detectable E_2 and T from trout and flounder plasma, or all E_2 from bream plasma. This residual steroid, coupled with the substantial dissociation of

bound ^{3}H-E_2 from trout, bream, flounder and snapper SBP in less than 5 min (Chapter 3, this volume), suggests some steroid may be irreversibly bound to SBP. This steroid may not dissociate in the changing equilibrium conditions of prolonged DCC incubation. Alternatively, another equilibrium may be reached with some steroid remaining bound to SBP. It is unlikely there was a problem with the performance of the DCC, as the same DCC protocol is effective at removing more than 90 % of radiolabelled free steroids in RIAs used routinely in our laboratory. This is in contrast to human SBP where more than 99 percent of endogenous steroids were removed by DCC (Rosner *et al.*, 1974). However, the results in our study suggest that B_{max} may be underestimated in some studies where endogenous steroids are not removed, or the effectiveness of the DCC stripping procedure is not confirmed by another method (Foucher *et al.*, 1992; McPherson, *et al.*, 1988; Pottinger, 1988; Pasmanik and Callard, 1986). If steroid is irreversibly bound to some sites, dilution is unlikely to remove it. In spite of this incomplete removal of endogenous steroid, a DCC stripping procedure was incorporated for the present study, as about half of the steroid measured in unstripped plasma by RIA was removed by DCC. The values reported here for B_{max}, however, may be underestimated to some extent.

Low Affinity Binding

The second component of binding apparent on some Scatchard plots was confirmed as an effect of a low affinity binder such as albumin or an albumin-like protein after denaturation of SBP by heating (Westphal, 1971). The binding remaining after heating was not saturable at steroid levels used in these assays and was a very small component of the total binding at all but the highest labelled steroid concentrations. The binding of ^{3}H-E_2 to the low affinity component appeared to account for the slightly upward-curved saturation plots of specific binding for the controls for trout, bream and snapper, however, there was little effect of the low affinity binder on the saturation curve for specific binding in flounder plasma. Both k_D and B_{max}, however, may be underestimated in the presence of low affinity binding such as albumin (Clark and Peck, 1979). Albumin in mammals is the most abundant plasma protein with the functions of binding of fatty acids and osmotic regulation (Kragh-Hansen, 1981). The presence of a plasma protein identical to mammalian albumin has not been confirmed in fishes. A protein binding free fatty acids with a molecular mass and isoelectric point similar to human albumin was absent from the plasma of common

carp *Cyprinus carpio* (De Smet *et al.*, 1998). However, albumin-like proteins binding palmitate have been found in both rainbow trout (Maillou and Nimmo, 1993a) and Atlantic salmon *Salmo salar* (Maillou and Nimmo, 1993b). If these proteins are also present in the plasma of bream, snapper and flounder, and they bind E_2 as mammalian albumin does, there may be interference in these SBP binding assays depending on several factors. If the concentration of the protein is sufficiently high, there may be measurable binding of E_2. However, the albumin-like protein in the serum of female Atlantic salmon comprised less than 10 % of the total serum protein, compared to greater than 50 % in males (Maillou and Nimmo, 1993b), therefore effects may have been minimised by the use of female fish in the present study. Interference in the assays is also dependant on the k_D of any albumin-like protein for E_2. If the k_D for E_2 is low compared to that of the SBP as it is in mammals (eg. k_D of human albumin for E_2 is around 1×10^{-5} M; Kragh-Hansen, 1981), then most binding to albumin will have dissociated during the incubation time with DCC in these assays as a separation time of less than 0.01 seconds is required to measure binding with a k_D of 10^{-6} M before substantial dissociation (Bennett, 1978).

2.5 References

Barnett, C.W. (1998). Reproductive biology and endocrinology of the greenback flounder *Rhombosolea tapirina* (Günther 1862). PhD thesis, University of Tasmania, Launceston.

Bennett, J.P. (1978). Methods in binding studies. *In* "Neurotransmitter Receptor Binding" (H.I. Yamamura, S.J. Enna, and M.J. Kuhar, Eds.), pp. 57-90. Raven Press, New York.

Chamness, G.C., and McGuire, W.L. (1975). Scatchard plots: common errors in correction and interpretation. *Steroids* **26**, 538-542.

Chang, C.F., and Lee, Y.H. (1992). Purification of the sex steroid-binding protein from common carp (*Cyprinus carpio*) plasma. *Comp. Biochem. Physiol.* **101B**, 587-590.

Clark, J.H., and Peck, E.J. (1979). "Female Sex Steroids: Receptors and Function", p. 245. Springer-Verlag, Berlin.

Cleary, J.J. (1997). The effect of stress on reproduction in snapper, *Pagrus auratus* (Sparidae). PhD thesis, University of Tasmania, Launceston.

De Smet, H., Blust, R., and Moens, L. (1998). Absence of albumin in the plasma of the common carp *Cyprinus carpio*: binding of fatty acids to high density lipoprotein. *Fish Physiol. Biochem.* **19**, 71-81.

Foucher, J.L., Le Bail, P.-Y., and Le Gac, F. (1992). Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **85**, 101-110.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Haddy, J.A., and Pankhurst, N.W. (1999). Stress-induced changes in concentrations of plasma sex steroids in black bream, *Acanthopagrus butcheri. J. Fish Biol.* **55**, 1304-1316.

Heyns, W. (1986). Measurement of steroid binding proteins by steroid binding. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 181-189. John Libbey Eurotext, Paris.

Hobby, A.C., and Pankhurst, N.W. (1997). Comparative aspects of steroid binding protein (SBP) in four fishes: a salmonid, a pleuronectid and two sparids. *In* "Advances in Comparative Endocrinology" (S. Kawashima, and S. Kikuyama, Eds.), pp. 1369-1373. Monduzzi Editore, Bologna.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1986). Specific binding of human corticosteroid-binding globulin to cell membranes. *Proc. Natl. Acad. Sci. USA* **83**, 3253-3256.

Idler, D.R., and Freeman, H.C. (1973). Stability to freezing of steroid binding proteins in fish plasma. *Comp. Biochem. Physiol.* **44B**, 179-183.

Kenakin, T.P. (1993). Radioligand binding experiments. *In* "Pharmacologic Analysis of Drug-Receptor Interaction" 2nd edn, pp. 385-410. Raven Press, New York.

Kragh-Hansen, U. (1981). Molecular aspects of ligand binding to serum albumin. *Pharm. Rev.* **33**, 17-53.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Laidley, C.W., and Thomas, P. (1997). Changes in plasma sex steroid-binding protein levels associated with ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **56**, 931-937.

Lindstedt, G., Lundberg, P., Hammond, G.L., and Vihko, R. (1985). Sex hormone-binding globulin - still many questions. *Scand. J. Clin. Lab. Invest.* **45**, 1-6.

Maillou, J., and Nimmo, I.A. (1993a). Albumin-like proteins in the serum of rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **104B**, 387-393.

Maillou, J., and Nimmo, I.A. (1993b). Identification and some properties of an albumin-like protein in the serum of pre-spawning Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol.* **104B**, 401-405.

Martin, B. (1975). Steroid-protein interactions in nonmammalian vertebrates. I. Elasmobranch steroids-binding protein (E.SBP) in dogfish serum (*Scyliorhinus canicula*). *Gen. Comp. Endocrinol.* **25**, 42-51.

Martin, B., and Ozon, R. (1975). Steroid-protein interactions in nonmammalian vertebrates. II. Steroids binding proteins in the serum of amphibians; a physiological approach. *Biol. Reprod.* **13**, 371-380.

McPherson, G.A. (1983). A practical computer based approach to the analysis of radioligand binding experiments. *Computer Prog. Biomed.* **17**, 107-114.

McPherson, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacological Methods* **14**, 213-228.

McPherson, R., Hannum, J., and Greco, T. (1988). An investigation of an estrogen-binding component in the liver and plasma of brook char, *Salvelinus fontinalis*. *Comp. Biochem. Physiol.* **89A**, 615-619.

Munson, P.J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.

Pankhurst, N.W., and Conroy, A.M. (1987). Seasonal changes in reproductive condition and plasma levels of sex steroids in the blue cod, *Parapercis colias* (Bloch and Schneider) (Mugiloididae). *Fish Physiol. Biochem.* **4**, 15-26.

Paolucci, M., and Di Fiore, M.M. (1994). Sex steroid binding proteins in the plasma of the green frog, *Rana esculenta*: Changes during the reproductive cycle and dependence on pituitary gland and gonads. *Gen. Comp. Endocrinol.* **96**, 401-411.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1988). Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **70**, 334-344.

Pottinger, T.G., and Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **80**, 264-273.

Rosner, W., Toppel, S., and Smith, R.N. (1974). Testosterone-estradiol-binding globulin of human plasma: denaturation and protection. *Biochim. Biophys. Acta* **351**, 92-98.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660-672.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., and Kuhn, R.W. (1982). The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38**, 457-510.

Thomas, P.M., Pankhurst, N.W., and Bremner, H.A. (1999). The effect of stress and exercise on post-mortem biochemistry of Atlantic salmon and rainbow trout. *J. Fish Biol.* **54**, 1177-1196.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

Westphal, U. (1971). "Steroid-Protein Interactions", p. 555. Monographs on Endocrinology 4, Springer-Verlag, New York.

Westphal, U. (1986). "Steroid-Protein Interactions II", p. 592. Monographs on Endocrinology 27, Springer-Verlag, New York.

Zeginiadou, T., Kolias, S., Kouretas, D., and Antonoglou, O. (1997). Nonlinear binding of sex steroids to albumin and sex hormone binding globulin. *Eur. J. Drug Metab. Pharmacokinet.* **22**, 229-235.

3. A comparison of sex steroid binding protein (SBP) in four species of teleost fish

3. A comparison of sex steroid binding protein (SBP) in four species of teleost fish.

3.1 Introduction

Steroid hormones circulate in the plasma of vertebrates either as free steroid, steroid bound to low specificity, high capacity binders such as albumin, or steroid bound to a specific steroid binding protein. Corticosteroids and progesterone are bound to a corticosteroid binding globulin (CBG) and androgens and estrogens are bound to a sex steroid binding protein (SBP). SBP is characterised by high affinity, moderate capacity binding of 17β-estradiol (E_2) and testosterone (T), and also the ability to bind other steroids (primarily other androgens and estrogens) with low to moderate affinity. Steroid bound to SBP is protected from metabolism, and in this way SBP acts as a plasma reservoir of T and/or E_2 (Callard and Callard, 1987). Receptors for SBP and CBG on cell membranes in humans (Hryb *et al.*, 1990) and internalisation SBP into human breast cancer cells (Porto *et al.*, 1991) suggest this binding protein is also directly involved in transport of steroids into some cells (Siiteri *et al.*, 1982).

Most information on SBP and its function is derived from mammalian studies (reviewed in Petra, 1991; Joseph, 1994), however, SBP has been detected in a number of teleosts including Atlantic cod *Gadus morhua* (Freeman and Idler, 1971), goldfish *Carassius auratus* (Pasmanik and Callard, 1986), carp *Cyprinus carpio* (Chang and Lee, 1992), spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1994) and the salmonids rainbow trout *Oncorhynchus mykiss* (Fostier and Breton, 1975), brown trout *Salmo trutta* (Pottinger, 1988), brook char *Salvelinus fontinalis* (McPherson, *et al.*, 1988) and Atlantic salmon *Salmo salar* (Freeman and Idler, 1971).

Circulating levels of steroids can vary by orders of magnitude between fish groups. Salmonids for example, generally have high plasma concentrations of steroids, with peak levels of E_2 and T around 50 ng ml^{-1} (180 nM) and 200 ng ml^{-1} (690 nM) respectively, during reproductive development in the rainbow trout (*O. mykiss*) (Scott *et al.*, 1980). In contrast, the sparid, snapper (= red sea bream *Pagrus auratus*), as an example at the other end of the spectrum, has low peak levels of reproductive steroids with maxima of 2.7 and

0.6 ng ml^{-1} (10 and 2.1 nM) for E_2 and T respectively (Hobby and Pankhurst, 1997a). It has been established that SBP characteristics differ between species. However, comparisons between studies utilising different methods are difficult. The aim of the present study was to establish whether SBP characteristics in species with 'low' circulating E_2 and T levels were consistently different from SBP characteristics in a species with considerably higher typical plasma steroid concentrations.

To address this, SBP was investigated in four species of fish; a pleuronectid - the greenback flounder (*Rhombosolea tapirina*), two sparids - snapper (*P. auratus*) and black bream (*Acanthopagrus butcheri*) and a salmonid, the rainbow trout (*O. mykiss*). Greenback flounder is a species with low to intermediate peak plasma levels of E_2 and T (3 ng ml^{-1} and 8 ng ml^{-1} (11 and 28 nM) respectively) in vitellogenic fish (Barnett and Pankhurst, 1999). Snapper have low peak levels of E_2 and T as mentioned previously (Hobby and Pankhurst, 1997a), as do black bream with peak plasma concentrations of 4.2 ng ml^{-1} and 2.9 ng ml^{-1} (15 and 10 nM) for E_2 and T respectively in reproductive fish (Haddy and Pankhurst, 1998). In addition to providing a species at the 'high' end of the spectrum of absolute plasma steroid levels (Scott *et al.*, 1980), rainbow trout served as a useful check on technique. The presence of SBP with high affinity but low capacity has already been established in rainbow trout (Fostier and Breton, 1975; Foucher *et al.*, 1992). In the present study, a charcoal adsorption binding assay was used to measure the apparent equilibrium dissociation constant as an indication of binding affinity, and the concentration of binding sites, for comparison of SBP between the four species.

3.2 Methods

Plasma Collection

Trout, flounder, bream and snapper were maintained, or captured as described in Thomas *et al.* (1999), Barnett and Pankhurst (1999), Haddy and Pankhurst (1998) and Cleary (1997) respectively. Blood samples were taken from female fish by caudal puncture with heparinised 22G needles. Blood samples were centrifuged and the plasma removed and stored at -20 °C. The female trout were reproductively immature. Female flounder of all reproductive stages from regressed to ovulated were represented in the pools of flounder

plasma. Bream ranged from regressed to final oocyte maturation stages, and snapper were either regressed or vitellogenic stage females.

Binding Assay

Before assay, plasma from 3 or more fish was pooled and then stripped of endogenous steroids by incubation with an equal volume of 20 $mg.ml^{-1}$ DCC for 20 min, with initial vortexing and then vortexing again after 10 min, followed by centrifugation (2060 g for 10 min). The supernatant was decanted and diluted for use in the assay. Assays were performed at plasma dilutions of 200-fold for trout, 100-fold for bream and 50-fold for flounder and snapper on the basis of preliminary experiments examining the effect of plasma dilution (Hobby and Pankhurst, 1997b; Chapter 2, this volume).

The hot saturation charcoal adsorption binding assay used has been previously described (Hobby and Pankhurst, 1997b; Chapter 2, this volume). In brief, DCC-stripped plasma was diluted in phosphate buffer and incubated with a range of concentrations of radio-labelled E_2 ([2,4,6,7-^{3}H]estradiol (Amersham) and [2,4-^{3}H]estradiol (Sigma)); 0.05-20 nM for trout, and 1-20 nM for bream and snapper, in the presence and absence of 100-fold excess of unlabelled E_2 as competitor. Assays of flounder plasma for low affinity binding were as above with 5-40 nM ^{3}H-E_2 and a plasma dilution of 20-fold, but this protocol was subsequently changed because the specific binding component in the plasma was not saturated with this range of ^{3}H-E_2 concentrations. For estimation of k_D and B_{max}, DCC-stripped, 50-fold diluted flounder plasma was incubated with 5 nM ^{3}H-E_2 and increasing concentrations (5-2500 nM) of unlabelled E_2. Tubes were equilibrated 30 min at room temperature, then overnight at 4 °C. The incubation was terminated by addition of 0.5 ml of 10 $mg.ml^{-1}$ DCC. Tubes were vortexed and incubated at 4 °C for 5 min prior to centrifugation at 2060 g and 4 °C for 10 min. The supernatant was decanted, 5 ml of Ecolite scintillation cocktail (ICN Biochemicals) added and vials were shaken and counted using a Beckman liquid scintillation counter.

The equilibrium dissociation constant (k_D), maximum number of binding sites (B_{max}), contribution of non-specific binding, and the number of binding components was determined using the computer programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980).

Association and Dissociation Kinetics

Diluted DCC-stripped plasma was incubated at 4 °C with 20 nM ^{3}H-E_2, with or without 100-fold excess of unlabelled competitor, for times ranging from 10 s to 24 h to determine the rate of association of E_2 to SBP. The incubation was terminated by addition of 0.5 ml 10 mg.ml^{-1} DCC. Plasma for investigation of the rate of dissociation was incubated with 20 nM ^{3}H-E_2 for 24 h (to equilibrium), followed by addition of 100-fold excess of unlabelled competitor for times ranging from 10 s to 24 h, before addition of DCC. Concerns about dissociation occurring during the 5 min incubation time with DCC resulted in repeating the assays with a protocol similar to that used by Foucher and Le Gac (1989) and Johnson *et al.* (1985). In this case, 100 µl of plasma diluted 200-fold in Tris buffer (0.05 M adjusted to pH 8 with 1 M HCl) were incubated with 150 µl of washed DEAE-biogel (Bio-Rad) (diluted 1:2 in Tris buffer) and 300 µl Tris buffer. After incubation for 45 min at 4 °C with gentle shaking every 10 min, the tubes were centrifuged at 1000 G for 5 min at 4 °C, and the supernatant was discarded. 150 µl of 20 nM ^{3}H-E_2 and 150 µl of Tris were added to each tube, except 3 tubes for non-specific binding with 150 ml of 100x excess competitor instead of Tris. The tubes were shaken gently and incubated overnight at 4 °C. Dissociation was induced by the addition of 20 µl of 64 µM E_2 competitor at times ranging from 15 s to 60 min. The assay was terminated by addition of 3 ml Tris buffer and suction under vacuum through Whatman GF/B glass fibre filter paper in a 48 well Cell Harvester (Brandel Biomedical Research and Development Laboratories Inc., Gaithersburg, MD 20877, USA). The tubes were washed 3 times with 3 ml Tris and the filter paper left to dry under vacuum for approximately 5 min. Ecolite scintillation cocktail was added to each filter paper in a 20 ml scintillation vial, and vials were left to sit for at least 3 h before vortexing and counting. The dissociation rate constant (k_{-1}) and the possible number of binding sites was determined using the computer program KINETIC (McPherson, 1985).

3.3 Results

Binding Characteristics

Binding affinity and capacity varied between the species. The highest affinity of SBP for ^{3}H-E_2 was observed in trout (k_D = 0.44 nM), followed by bream (3.39 nM), then snapper (10.72 nM) and the lowest binding affinity for ^{3}H-E_2 was observed with plasma from

flounder (84.7 nM) (Table 3.1). The concentration of binding sites was highest in flounder (164 nM), followed by trout (92 nM), and then bream (50 nM) and lowest in snapper (39 nM) (Table 3.1). Specific binding was saturated at ligand concentrations used in the assays (Fig. 3.1). Only a single binding site for E_2 was detected in each species using saturation assays (Fig. 3.2). Despite some curvature of the Scatchard plots for trout and flounder (Fig. 3.2), fitting a model in LIGAND with 2 binding sites did not significantly improve the fit of the model.

Association and Dissociation Kinetics

For all species, binding of ^{3}H-E_2 to SBP was rapid and appeared to remain stable for up to 24 h (Fig. 3.3). Dissociation of the complex in the presence of 100-fold excess competitor was also rapid with most dissociation occurring in the first 5 min after addition of competitor for all 4 fish species (Fig. 3.3). Binding of ^{3}H-E_2 in trout, flounder and snapper appeared to consist of 2 sites, with the first site accounting for more than 50 % of the binding, and the second site comprising between 33 and 44 % of binding (Table 3.2). All binding of ^{3}H-E_2 to bream plasma was accounted for by a single binding site. The rate of dissociation from the first binding site in trout and flounder plasma was extremely rapid ($k_{-1} > 3$ for both species) (Table 3.2). Dissociation was slower in bream and snapper ($k_{-1} = 0.12$ and 0.57 for bream and snapper respectively).

Specific binding at t_0 of dissociation ranged from 61 to 86 %, and was reduced to 23 to 37 % after 5 min of dissociation in trout, bream and snapper (Table 3.3). Specific binding in flounder declined from 85 % at t_0 to 11 % after 5 min dissociation (Table 3.3). Measurement of specific binding from the DCC-adsorption assays in Table 3.1 (ie. after 5 min incubation with DCC) ranged from 44 to 55 % (Table 3.3).

3.4 Discussion

Estimates of k_D varied widely between species. Trout SBP had the lowest k_D for E_2 in the present study, followed by bream and then snapper SBP. The k_D of flounder SBP for E_2 was highest at around 200x more than that of trout SBP. The k_D values for E_2 determined

Table 3.1: Values of k_D and B_{max} from 3 pools of plasma, and maximum measured E_2 and T concentrations (nM) for each species.

	Mean k_D (nM) (95 % CL)	Mean B_{max} (nM) (95 % CL)	E_2 (nM)	T (nM)	Reference for steroid levels
trout	0.44 (0.33, 0.58)	92 (83, 101)	110	485	Pankhurst and Thomas 1998
flounder	84.7 (56.1, 128.0)	164 (124, 216)	11	28	Barnett and Pankhurst 1999
bream	3.39 (1.67, 6.85)	50 (31, 78)	15	10	Haddy and Pankhurst 1998
snapper	10.7 (6.8, 16.8)	39 (27, 55)	~10	~2.1	Hobby and Pankhurst 1997a

Figure 3.1: Representative saturation plots for trout, flounder, bream and snapper. The plots for trout, bream and snapper showing total (hollow circle), specific (filled circle) and non-specific (hollow square) binding. The saturation plot for flounder shows only specific binding as non-specific binding was measured at the lowest ^{3}H-E_2 concentration only.

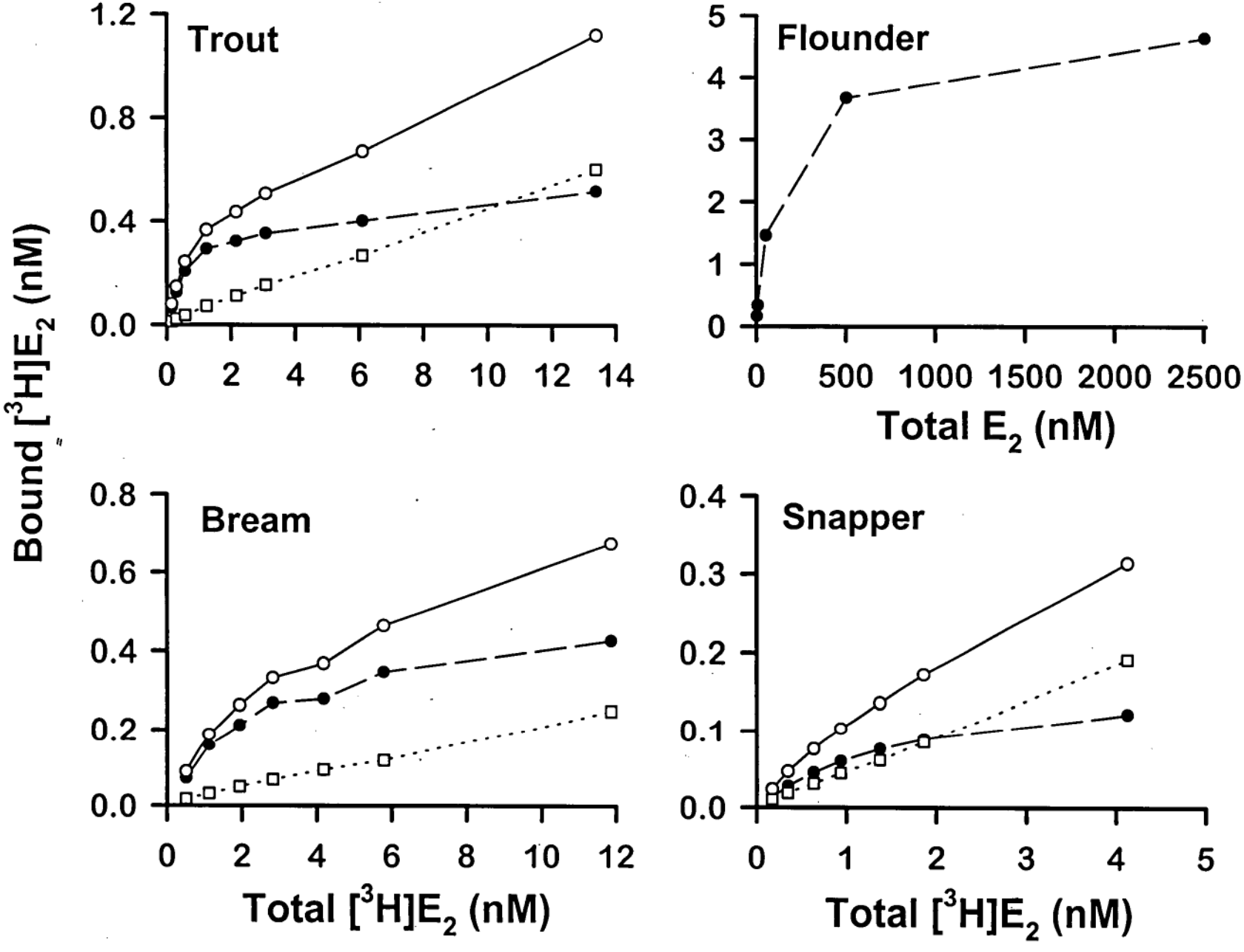

Trout
Flounder
Bream
Snapper
Bound [3H]E2 (nM)
Total E2 (nM)
Total [3H]E2 (nM)
Total [3H]E2 (nM)
0.0
0.4
0.8
1.2
0
2
4
6
8
10
12
14
0
1
2
3
4
5
0
500
1000
1500
2000
2500
0.0
0.2
0.4
0.6
0.8
0
2
4
6
8
10
12
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5

Figure 3.2: Representative Scatchard plots for trout, flounder, bream and snapper.

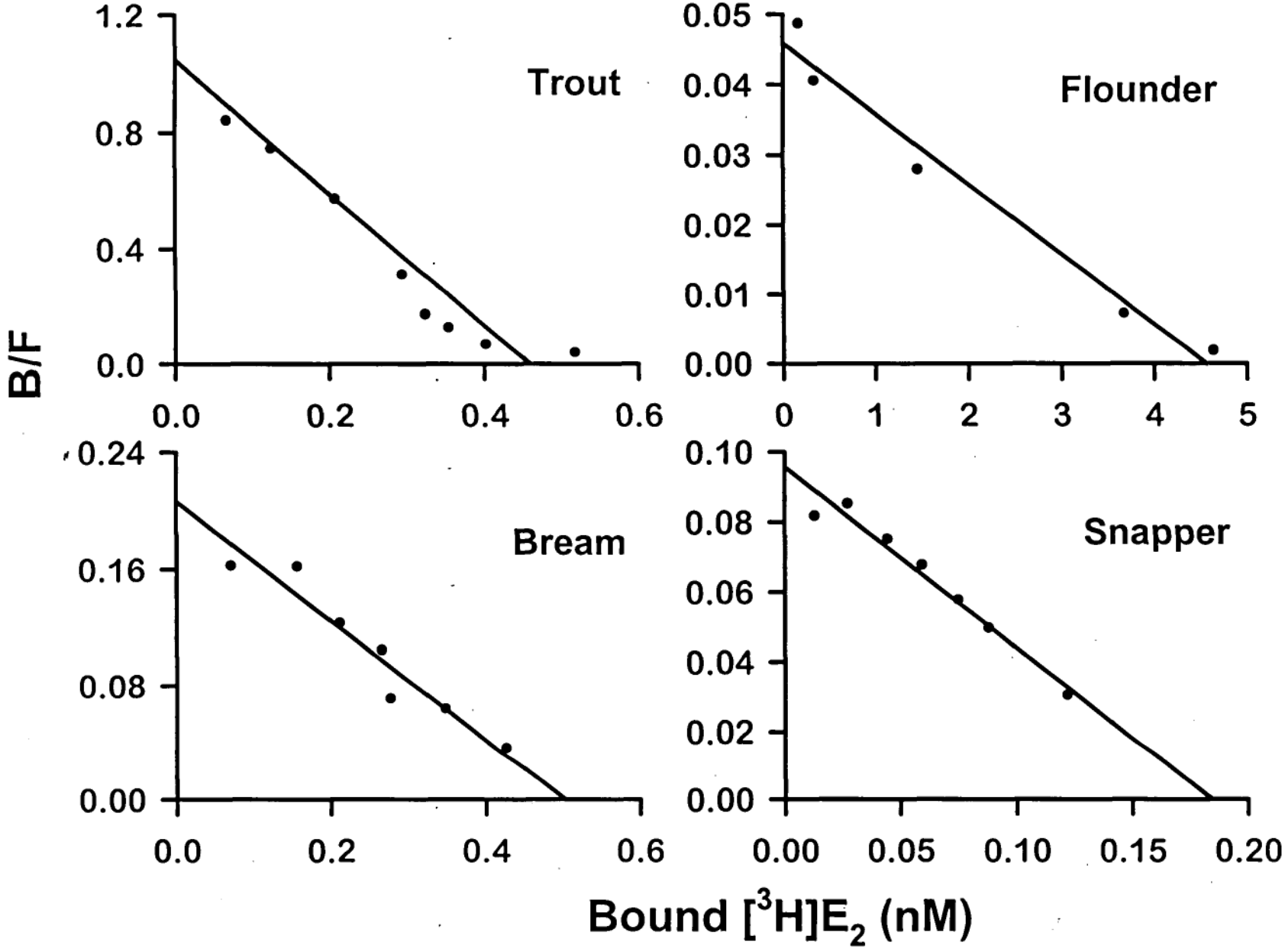

Trout
Flounder
Bream
Snapper
B/F
Bound [3H]E2 (nM)
1.2
0.8
0.4
0.0
0.0
0.2
0.4
0.6
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
4
5
0.24
0.16
0.08
0.00
0.0
0.2
0.4
0.6
0.10
0.08
0.06
0.04
0.02
0.00
0.00
0.05
0.10
0.15
0.20

Figure 3.3: Association kinetics (closed circles) (measured with the DCC-adsorption method and allowing for continued association during the 5 min incubation time with DCC), and dissociation kinetics (open circles) (measured with the DEAE-biogel filtration method) for trout, flounder, bream and snapper plasma SBP at 4 °C.

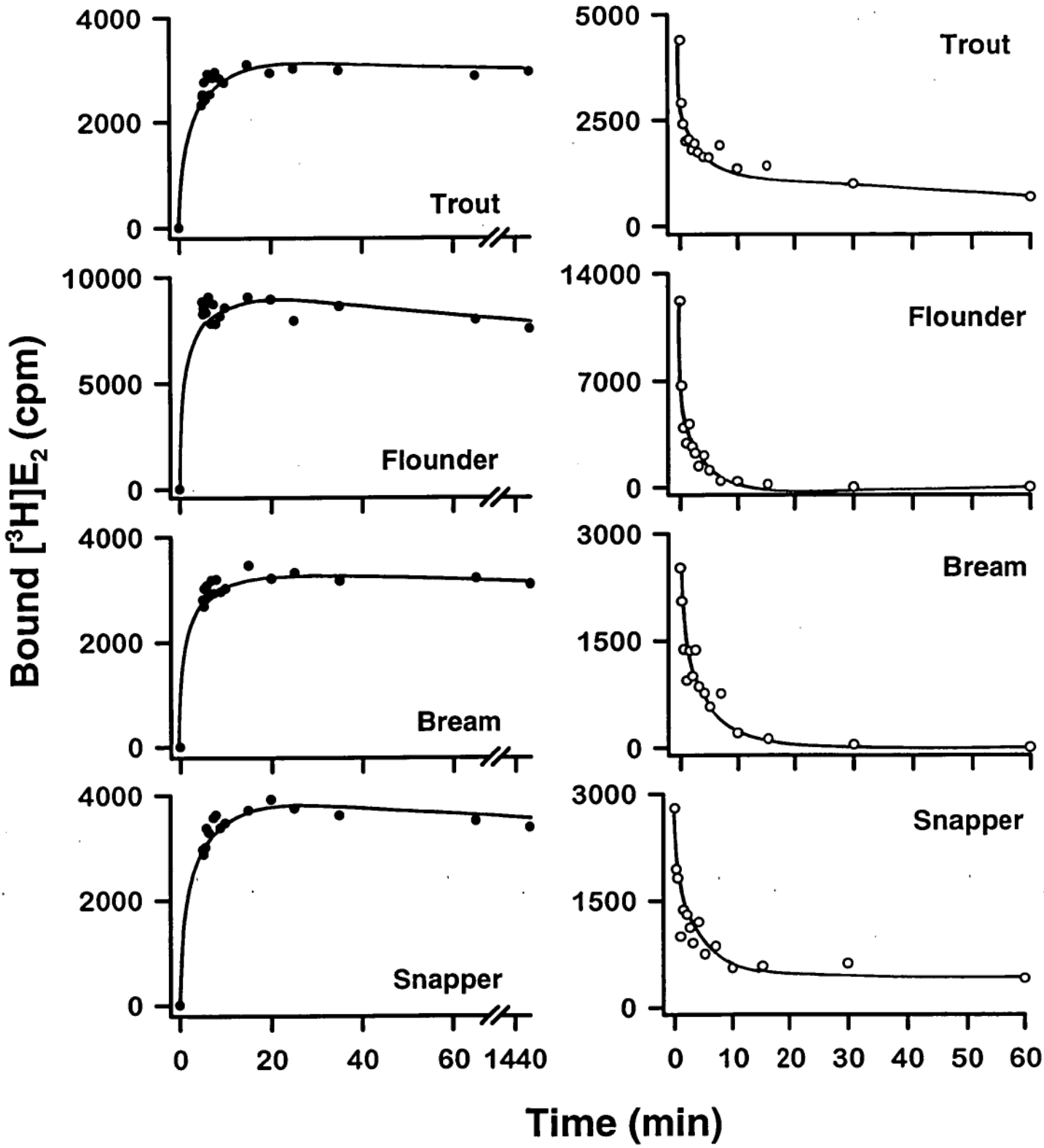

Bound [^{3}H]E$_2$ (cpm)
4000
2000
0
Trout
10000
5000
0
Flounder
4000
2000
0
Bream
4000
2000
0
Snapper
0
20
40
60
1440
5000
2500
0
Trout
14000
7000
0
Flounder
3000
1500
0
Bream
3000
1500
0
Snapper
0
10
20
30
40
50
60
Time (min)

Table 3.2: Dissociation rate constants (k_{-1}) and percent of binding accounted for by each binding site, and associated percent errors (from KINETIC, in brackets) for trout, flounder, bream and snapper, measured by the DEAE-biogel filtration method.

	k_{-1} site 1	k_{-1} site 2	Binding to site 1 at t_0 (%)	Binding to site 2 at t_0 (%)
Trout	3.03 (23)	0.02 (9)	56 (15)	44 (3)
Flounder	4.67 (56)	0.20 (12)	70 (43)	30 (16)
Bream	0.12 (5)		100 (10)	
Snapper	0.57 (35)	0.01 (45)	65 (20)	35 (15)

Table 3.3: Specific binding before, and after 5 min dissociation measured with the DEAE-biogel filtration method, and specific binding measured from the DCC-adsorption assays in this chapter.

	SB t_0 (%) [1]	SB 5 min (%) [2]	SB 5 min corrected (%) [3]	SB DCC (mean ± se (n)) (%) [4]	SB DCC corrected (%) [5]
Trout	86	37	43	53 ± 4 (3)	62
Flounder	85	9	11	48 ± 3 (3)	56
Bream	61	23	38	55 ± 6 (3)	90
Snapper	61	27	44	44 ± 4 (3)	72

[1] Specific binding at t_0 of dissociation.

[2] Specific binding remaining after 5 min dissociation.

[3] Specific binding remaining after 5 min dissociation as a percentage of specific binding at t_0.

[4] Specific binding measured from DCC-adsorption assays.

[5] Specific binding measured from DCC-adsorption assays as a percentage of specific binding at t_0.

in the present study are within the range reported for other teleosts such as goldfish (2.1 nM; Pasmanik and Callard, 1986), Atlantic salmon (13 nM; Lazier *et al.*, 1985), spotted sea trout (3.1 nM; Laidley and Thomas, 1994) and brown trout (48 nM; Pottinger, 1986). However, the binding affinity for E_2 of flounder SBP (85 nM) is lower than that measured for other species. This k_D is at the low end of "high affinity" binding. The possibility that flounder SBP was a less specific form of SBP was investigated in Chapter 4, this volume.

Binding capacity also varied with species, with the highest capacity for E_2 found in flounder, followed by trout. The capacity of bream plasma to bind E_2 was about 30 %, and snapper approximately 20 % of the capacity of flounder plasma. The binding capacities measured in the present study parallel the peak levels of steroids seen in these fish if flounder is excluded. Rainbow trout has higher peak plasma concentrations of E_2 and T and also higher B_{max} than bream and snapper. Differences between the phylogenetically similar bream and snapper appear to be minimal (B_{max} differs by less than 10 %). However, trout also have substantially higher steroid levels than flounder, but the B_{max} of flounder SBP is higher. There is thought to be a general trend of decreasing binding capacity and circulating levels of steroids with more phylogenetically 'modern' animals (Callard and Callard, 1987). This trend is followed by trout, snapper and bream with higher capacity binding in the phylogenetically more primitive salmonid, but binding in flounder does not fit this pattern.

The binding characteristics of trout SBP measured in the current study differ from those found in other studies. The affinity of rainbow trout SBP for E_2 measured in the present study is higher than has been reported previously (k_D = 0.44 nM, the present study; versus k_D = 4.7 nM for T, Foucher *et al.*, 1991; $k_A = 1.8 \times 10^8\ M^{-1}$ ($k_D \sim 5.5$ nM) for E_2, Fostier and Breton, 1975; and k_D = 16.8 nM for E_2, Pottinger and Pickering, 1990). The binding capacity of trout SBP measured in the present study was lower than previously reported values (92 nM versus 2000 nM (Fostier and Breton, 1975) and ~400 nM (Foucher *et al.*, 1991)). The most likely explanation is the different assay protocols (hot or cold saturation) and different methods used to separate bound and free steroid. A hot saturation assay was used in the present study, and DCC adsorption with a 5 min incubation time was used to separate bound and free steroid. A cold saturation protocol and equilibrium dialysis were used by Fostier and Breton (1975), a cold saturation protocol and incubation for 15 min

with DCC was used by Pottinger and Pickering (1990), and a centrifugation/filtration method, also with a cold saturation protocol was used by Foucher *et al.* (1991). Overestimation of B_{max} and underestimation of binding affinity (overestimation of k_D) may occur with a cold saturation protocol. Slaunwhite and Rosenthal (1976) have also suggested that capacity measured by adsorption may be 40 % lower than that measured by equilibrium dialysis. Measurement of SBP by the charcoal adsorption method has also been compared to other methods such as equilibrium dialysis by Mercier-Bodard *et al.* (1970), and similar results were obtained from all methods. However, those authors used a very short charcoal incubation time of 15 s to adsorb free steroid, which would reduce the number of tubes able to be processed using the DCC-adsorption protocol from the current study. Mercier-Bodard *et al.* (1970) also used a short centrifuge time of 1 min. No differences were found in the present study using centrifuge times from 2 to 15 min (Chapter 2, this volume). Presumably the separation of DCC from the supernatant occurs relatively quickly, and the pellet of DCC at the bottom of the test tube is less able to adsorb any more steroid from the supernatant. Underestimation of B_{max} resulting from the DCC-adsorption method presents problems when comparing binding characteristics between studies. In the present study, DCC-adsorption was used for separation of bound and free steroid for all species, so relative differences remain, despite the potential for underestimating binding capacity.

The rapid association rate of SBP for E_2 at 4 °C ($t_{½} < 5$ min) for the four species examined here is consistent with the suggested role of SBP as a mechanism to protect steroid from degradation in the plasma. Rapid association rates have also been reported for the spotted seatrout ($t_{½} < 30$ s at 4 °C, Laidley and Thomas, 1994) and $t_{½} < 5$ min at 4 °C for the goldfish, dogfish *Squalus acanthias* and alligator *Alligator mississippiensis* (Pasmanik and Callard, 1986; Ho *et al.*, 1980, 1987). The extremely rapid half-time of binding of progesterone to the progesterone binding globulin of the pregnant guinea pig (22.5 ms at 20 °C, Westphal, 1978) suggests that association of E_2 to SBP may be even quicker than described here, but the limitations of our technique prevented detection of possible shorter association times.

The dissociation curves for all 4 species show most dissociation occurring in the first 5 min after addition of excess competitor. Dissociation rates for ^{3}H-E_2 from goldfish SBP and

^{3}H-T from spotted seatrout SBP are also very rapid with $t_{½} < 5$ min (Pasmanik and Callard, 1986; Laidley and Thomas, 1994). Similar rates of dissociation of E_2 and T have been reported for the green frog *Rana esculenta* and lizard *Podarcis s. sicula* (Paolucci and Di Fiore, 1994; Paolucci *et al.*, 1992). Dissociation half-times from human SBP are also in the same range, at 3.3 min for ^{3}H-E_2 and 0.22 to 12.9 min for ^{3}H-T (reviewed in Westphal, 1986). The second binding site apparent from the dissociation kinetics in trout, flounder and snapper is likely to be non-specific or 'albumin-type' binding that could have been removed if non-specific binding had been measured at each dissociation time. The fact that dissociation of bound ligand from bream SBP was completely accounted for by a single binding site appears to reflect that binding to other proteins including albumin-like proteins was accounted for in the non-specific binding fraction in these assays. It therefore appears that binding to SBP is wholly and very rapidly reversible. A very fast half time of dissociation would allow substantial transfer of steroid from SBP to receptors while in the target organ.

The differing rates of dissociation between the species may present a problem for determination of B_{max} using a 5 min incubation time with DCC to separate bound and free steroid as in the present study. Use of the DEAE-biogel/filtration method to investigate dissociation kinetics allowed separation of bound and free steroid in under 10 s. Specific binding at t_0 of dissociation ranged from 61 to 86 %. Specific binding after 5 min of dissociation was considerably lower, with only 11 to 44 % of the initial level of specific binding remaining. This suggests that underestimation of B_{max} using a 5 min incubation time with DCC will occur, and also that the level of specific binding measured in assays using the DCC method should be quite low. However, specific binding measured for the assays in Table 3.1 ranged from 44 to 55 %. If this level of specific binding is corrected for the initial level of specific binding measured at t_0 of dissociation (61 - 86 %), the specific binding remaining in the DCC assays is between 56 and 90 % of the specific binding at t_0. The B_{max} estimates in Table 3.1, corrected for the possible reduction in specific binding after 5 min dissociation are presented in Table 3.4. The relative differences between the B_{max} estimated for each species remain, regardless of the absolute value of B_{max}. The difference between specific binding remaining after 5 min dissociation, and specific binding measured in the DCC-adsorption assays suggests DCC is not as effective a

Table 3.4: Estimates of B_{max} corrected for underestimation due to dissociation during the 5 min incubation with DCC.

	B_{max} [1]	B_{max} corrected for the most likely case [2]	B_{max} corrected for the worst case [3]
Trout	94	152	219
Flounder	164	293	1491
Bream	50	56	132
Snapper	39	54	89

[1] B_{max} from Table 1.

[2] B_{max} from Table 1 corrected for underestimation assuming specific binding at t_0 has been reduced to 56 - 90 % of the initial level during incubation with DCC.

[3] B_{max} from Table 1 corrected for underestimation assuming specific binding at t_0 has been reduced to 11 - 44 % of the initial level during incubation with DCC.

competitor against SBP for the ^3H-E_2 ligand as 100x excess unlabelled E_2 is against ^3H-E_2 for SBP binding sites.

The potential underestimation of the B_{max} of flounder SBP is substantially larger than for the other species, and warrants greater concern. However, 2 assays of pools of flounder plasma were performed using a cold saturation protocol and the DEAE-biogel/filtration method as described by Foucher and Le Gac (1989). A difference in B_{max} with these assays would indicate the magnitude of the underestimation problem. B_{max} measured in this way, however, agreed very well with the uncorrected B_{max} estimate from the DCC-adsorption method (146 nM compared to 164 nM for the filtration and adsorption methods respectively). These preliminary results suggest substantial underestimation (ie. greater than 50 %) of B_{max} by the DCC method is unlikely. However, there was a difference in k_D measured with the two methods (k_D = 11 nM for the DEAE-biogel/filtration method and k_D = 85 nM for the DCC method). This provides further support for the first binding site with very rapid dissociation apparent from the dissociation kinetics, and suggests the k_D measured by the DCC adsorption method has been substantially influenced by the second binding site with slower dissociation. While ligands tend to dissociate from high affinity binding sites at a slower rate than lower affinity binding sites, the k_D is related to both the dissociation and association rates, suggesting the first binding site has very rapid association as well as dissociation. Use of DCC to adsorb free steroid results in a change of the equilibrium conditions as steroid is sequestered by DCC. Higher affinity binding sites tend to bind low concentrations of ligand, and these low ligand concentrations are likely to be sequestered by DCC more easily than higher ligand concentrations. This may also help explain why a lower k_D was measured using the filtration assay. Binding to more than 1 binding site may explain the discrepancies between studies, as relatively small differences in incubation time or the separation protocol will shift the assay to different points along the biphasic dissociation curve. This information also provides further evidence for the differences between SBP in the 4 species. SBP in flounder shows higher capacity for E_2 and T, but dissociation is very rapid. SBP in trout exhibits a very low k_D and B_{max} may or may not be exceeded by peak levels of E_2 and T at some stages of the reproductive cycle, and dissociation of E_2 from SBP is very rapid. Bream and snapper SBPs exhibit a similar k_D and B_{max}, and a slower (but still rapid) rate of dissociation of E_2.

The binding capacity of snapper, bream and flounder SBP measured in these assays is sufficient to bind all E_2 and T, even at the highest circulating levels that have been measured for these steroids. Measured plasma SBP levels also exceed circulating steroid levels in seatrout (Laidley and Thomas, 1994), goldfish (Pasmanik and Callard, 1986), Atlantic salmon, Atlantic cod and thorny skate *Raja radiata* (Freeman and Idler, 1971). It has been suggested that SBP concentrations may be high to facilitate steroid uptake in steroidogenic tissues rather than to meet requirements of plasma binding (Laidley and Thomas, 1994). In trout, however, the peak levels of E_2 and T may sometimes exceed the measured SBP capacity. Peak E_2 and T levels of 30 and 140 ng ml^{-1} (110 and 485 nM) respectively, have been measured in the rainbow trout population maintained at the University of Tasmania (Pankhurst and Thomas, 1998) which is greater than the SBP capacity measured in the present study. Steroid levels in excess of the capacity of SBP would alter the equilibrium between SBP-bound, 'albumin'-bound and free steroid. If, as in humans, about 70 percent of E_2 is bound to 'albumin' and only approximately 30 percent is available to bind to SBP, this is still nearly double the capacity of SBP, implying an increase in the proportion of steroid that is either free and/or bound to 'albumin'. This, in turn, implies a higher steroid concentration available for diffusion in target tissues but also a greater loss of steroid to metabolism/degradation. There is also the possibility that binding capacity varies on a seasonal basis in concert with reproductive state and steroid levels (Foucher *et al.*, 1992; Pottinger, 1988), and immature trout as used in this investigation may have lower binding capacity than mature trout. Foucher *et al.* (1992) found a decrease in SBP binding capacity in rainbow trout near the end of the reproductive cycle, and in brown trout plasma binding capacity is also lowest during the spawning period (Pottinger, 1988). The possible change in SBP binding characteristics with reproductive stage was investigated in Chapter 5, this volume.

The question addressed here was whether differences in SBP binding characteristics existed in these 4 species. It has been established that differences are present when the DCC-adsorption method is used. Differences are apparent in k_D and B_{max}. Investigation of the dissociation rate raised questions about whether the DCC method was appropriate to compare binding characteristics of different species. However, the different dissociation rates also provided further support of the differences between SBP. Correction of the B_{max} values by probable and 'worst case' levels of dissociation at 5 min (Table 3.3) does not

change either the relative B_{max} differences between the species, or the order from highest to lowest B_{max}. It is also unlikely that binding affinities measured for bream and snapper would change if another method was employed, whereas the data do suggest that if anything, the k_D measured for trout may be even lower than the estimate in the present study of 0.44 nM. Therefore, there are substantial differences in k_D between SBPs in trout, bream and snapper. Flounder SBP is in turn different again from that of trout, bream and snapper, in that the binding appears to be higher capacity, with an extremely rapid dissociation time such that high affinity binding is not apparent with the DCC method. In conclusion, differences in the SBP binding characteristics between the species are unable to be explained solely by different plasma steroid profiles. Phylogeny may play a role, along with other as yet unidentified factors in determining binding characteristics of SBP in these fish.

3.5 References

Barnett, C.W., and Pankhurst, N.W. (1999). Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther 1862). *Mar. Freshwater Res.* **50**, 35-42.

Callard, I.P., and Callard, G.V. (1987). Sex steroid receptors and non-receptor binding proteins. *In* "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D.O. Norris, and R.E. Jones, Eds.), pp. 355-384. Plenum Press, New York.

Chang, C.F., and Lee, Y.H. (1992). Purification of the sex steroid-binding protein from common carp (*Cyprinus carpio*) plasma. *Comp. Biochem. Physiol.* **101B**, 587-590.

Cleary, J.J. 1997. The effect of stress on reproduction in snapper, *Pagrus auratus* (Sparidae). PhD thesis, University of Tasmania, Launceston.

Fostier, A., and Breton, B. (1975). Binding of steroids by plasma of a teleost: the rainbow trout, *Salmo gairdnerii*. *J. Steroid Biochem.* **6**, 345-351.

Foucher, J.L., and Le Gac, F. (1989). Evidence for an androgen binding protein in the testis of a teleost fish (*Salmo gairdneri* R.): a potential marker of sertoli cell function. *J. Steroid Biochem.* **32**, 545-552.

Foucher, J.L., Niu, P.D., Mourot, B., Vaillant, C., and Le Gac, F. (1991). *In vivo* and *in vitro* studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Molec. Biol.* **39**, 975-986.

Foucher, J.L., Le Bail, P.-Y., and Le Gac, F. (1992). Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **85**, 101-110.

Freeman, H.C., and Idler, D.R. (1971). Binding affinities of blood proteins for sex hormones and corticosteroids in fish. *Steroids* **17**, 233-250.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Ho, S.M., Tsang, P., and Callard, I.P. (1980). Some properties of a steroid-binding protein in the plasma of an ovoviviparous dogfish, *Squalus acanthias*, at different stages of the life cycle. *Biol. Reprod.* **23**, 281-289.

Ho, S.M., Lance, V., and Megaloudis, M. (1987). Plasma sex-steroid binding protein in a seasonally breeding reptile, *Alligator mississippiensis*. *Gen. Comp. Endocrinol.* **65**, 121-132.

Hobby, A.C., and Pankhurst, N.W. (1997a). The relationship between plasma and ovarian levels of gonadal steroids in the repeat spawning marine fishes *Pagrus auratus* (Sparidae) and *Chromis dispilus* (Pomacentridae). *Fish Physiol. Biochem.* **16**, 65-75.

Hobby, A.C., and Pankhurst, N.W. (1997b). Comparative aspects of steroid binding protein (SBP) in four fishes: a salmonid, a pleuronectid and two sparids. *In* "Advances in Comparative Endocrinology" (S. Kawashima, and S. Kikuyama, Eds.), pp. 1369-1373. Monduzzi Editore, Bologna.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048-6054.

Johnson, A.R., Holmes, S.D., Lipshultz, L.I., and Smith, R.G. (1985). Rapid method for quantification of androgen binding protein in sertoli cell cultures and its use for measurement of binding kinetics. *J. Steroid Biochem.* **22**, 9-14.

Joseph, D.R. (1994). Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197-280.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Lazier, C.B., Lonergan, K., and Mommsen, T.P. (1985). Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. *Gen. Comp. Endocrinol.* **57**, 234-245.

McPherson, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacological Methods* **14**, 213-228.

McPherson, R., Hannum, J., and Greco, T. (1988). An investigation of an estrogen-binding component in the liver and plasma of brook char, *Salvelinus fontinalis*. *Comp. Biochem. Physiol.* **89A**, 615-619.

Mercier-Bodard, M., Alfsen, A., and Baulieu, E.E. (1970). Sex steroid binding plasma protein (SBP). *Acta Endocrinol. Suppl.* **147**, 204-224.

Munson, P.J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.

Pankhurst, N.W., and Thomas, P.M. (1998). Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. *Aquaculture* **166**, 163-177.

Paolucci, M., and Di Fiore, M.M. (1994). Sex steroid binding proteins in the plasma of the green frog, *Rana esculenta*: Changes during the reproductive cycle and dependence on pituitary gland and gonads. *Gen. Comp. Endocrinol.* **96**, 401-411.

Paolucci, M., De Fiore, M.M., Ciarcia, G., and Botte, V. (1992). Plasma sex steroid binding proteins (SSBP) in the male lizard, *Podarcis s. sicula*, during the reproductive cycle. *Gen. Comp. Endocrinol.* **87**, 232-239.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Molec. Biol.* **40**, 735-753.

Porto, C.S., Gunsalus, G.L., Bardin, C.W., Phillips, D.M., and Musto, N.A. (1991). Receptor-mediated endocytosis of an extracellular steroid-binding protein (TeBG) in MCF-7 human breast cancer cells. *Endocrinology* **129**, 436-445.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1988). Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **70**, 334-344.

Pottinger, T.G., and Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **80**, 264-273.

Scott, A.P., Bye, V.J., and Baynes, S.M. (1980). Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson). *J. Fish Biol.* **17**, 587-592.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., and Kuhn, R.W. (1982). The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38**, 457-510.

Slaunwhite, W.R., and Rosenthal, H. (1976). Quantitation of specific binding proteins. *In* "Hormones in Human Blood. Detection and Assay" (H.N. Antoniades, Ed.), pp. 777-794. Harvard University Press, Cambridge.

Thomas, P.M., Pankhurst, N.W., and Bremner, H.A. (1999). The effect of stress and exercise on post-mortem biochemistry of Atlantic salmon and rainbow trout. *J. Fish Biol.* **54**, 1177-1196.

Westphal, U. (1978). Steroid-binding serum globulins: Recent results. *In* "Receptors and Hormone Action" (B.W. O'Malley, and L. Birnbaumer, Eds.), pp. 443-472. Academic Press, New York.

Westphal, U. (1986). "Steroid-Protein Interactions II", p. 592. Monographs on Endocrinology 27, Springer-Verlag, New York.

4. Relative affinity of sex steroid binding protein (SBP) for a range of steroids in four teleost fish species

4. Relative affinity of sex steroid binding protein (SBP) for different steroids in four teleost fish species

4.1 Introduction

Sex steroid binding protein (SBP) is present in most animals including many mammals, reptiles, amphibians and fishes (Petra, 1991). Sex steroid binding protein (SBP) is a large molecule circulating in the plasma that protects estrogens and androgens (primarily E_2 and T) from metabolism, aids transport of these steroids to their target tissues and may also be involved in the passage of these steroids across the cell membrane (Hammond, 1990; Joseph, 1994). Other steroids also bind to SBP, but typically with lower affinity than E_2 and T, and this characteristic helps distinguish SBP from other binding proteins such as receptors which tend to be specific for a single ligand.

The affinity and maximum binding capacity of SBP for E_2 varies between the 4 fish species investigated here - a salmonid (rainbow trout *Oncorhynchus mykiss*), two sparids (black bream *Acanthopagrus butcheri* and snapper (= red sea bream) *Pagrus auratus*), and a pleuronectid (greenback flounder *Rhombosolea tapirina*) (Chapter 3 this volume). E_2 is bound to SBP with very high affinity in rainbow trout (k_D = 0.44 nM) and also in bream and snapper (k_D = 3.4 and 10.7 nM respectively). However, the affinity of flounder SBP for E_2 is much lower (k_D = 84.7 nM) (Chapter 3, this volume). Binding capacity bears some relation to maximum plasma E_2 and T levels in trout, bream and snapper. Snapper with the lowest circulating steroid levels has the lowest B_{max} (39 nM), whereas trout with the highest circulating steroid levels has a high B_{max} (92 nM). Flounder, however, does not fit this trend with moderate circulating plasma E_2 and T levels but the highest binding capacity (164 nM) (Chapter 3, this volume).

Among vertebrates there is a phylogenetic trend from a binding protein that shows low specificity in more primitive vertebrates, to separate binding systems for reproductive steroids and corticosteroids in higher vertebrates (Callard and Callard, 1987). For example, E_2, progesterone (P) and dihydrotestosterone (DHT) are bound to the SBP of an elasmobranch, the dogfish *Scyliorhinus canicula* with similar affinity, but other estrogens, androgens, progestogens and corticosteroids are also bound with more than 60 % of the

affinity of P (Martin, 1975). In teleosts, however, progestogens and corticosteroids are generally bound to SBP with lower affinity than estrogens and androgens (Fostier and Breton, 1975; Chang and Lee, 1992; Laidley and Thomas, 1994). There is also evidence for a second binding system preferentially binding corticosteroids and progestogens in rainbow trout (Fostier and Breton, 1975; Caldwell *et al.*, 1991). In reptiles such as the turtle *Chrysemys picta*, E_2, T and P are bound to the same protein with similar affinities (Salhanick and Callard, 1980). However, in amphibians and mammals, some androgens and estrogens are bound specifically to SBP, and there is negligible binding of cortisol and P, which are bound to corticosteroid-binding globulin (CBG) (Martin and Ozon, 1975; Renoir *et al.*, 1980; Paolucci and Di Fiore, 1994). If the phylogenetic trend holds within the teleosts, it might be expected that the SBP of the phylogenetically older rainbow trout (Nelson, 1984) would show lower specificity for steroids than the SBPs of bream, snapper and flounder.

SBP is characterised by high affinity, moderate capacity binding of T and E_2, and also the ability to bind other steroids (primarily other androgens and estrogens) with low to moderate affinity. To confirm that the plasma proteins binding E_2 measured in the present study were SBP, the relative binding affinity of the plasma protein for different steroids was measured in rainbow trout, greenback flounder, black bream and snapper. This enabled further assessment of the differences in binding characteristics between flounder and the other species, and investigation of the presence of a phylogenetic trend of binding specificity within teleosts. The steroids investigated included estrogens, androgens, some progestogens and cortisol. Binding of several conjugated forms of E_2 to SBP was also investigated to help determine which parts of the steroid structure are most important for high affinity binding by SBP.

4.2 Methods

The charcoal adsorption binding assay has been previously described (Chapter 2, this volume). In brief, dextran-coated charcoal (DCC) stripped plasma was diluted in phosphate buffer and incubated with a constant amount (5 nM) of radio-labelled E_2 ([2,4-^{3}H]estradiol (Sigma)) and increasing concentrations (5 nM - 50 μM) of competitor, (See Table 4.1 for a list of steroid competitors, including full names, common names and

Table 4.1: Steroids and conjugated steroids examined for specific binding to SBP.

Full name	Common name	Abbreviation
1,3,5[10]-estratriene-3,17β-diol	17β-estradiol	E_2
1,3,5[10]-estratrien-3-ol-17-one	estrone	E_1
1,3,5[10]-estratriene-3,16α,17β-triol	estriol	E_3
1,3,5[10]-estratriene-3,17β-diol 3-glucuronide	17β-estradiol 3-(β-$_D$-glucuronide)	E_2-3gluc
1,3,5[10]-estratriene-3,17β-diol 17-glucuronide	β-estradiol 17-(β-$_D$-glucuronide)	E_2-17gluc
1,3,5[10]-estratriene-3,17β-diol 3-glucuronide 17-sulfate	17β-estradiol 3-glucuronide 17-sulfate	E_2-3gluc-17sulf
4-androsten-17β-ol-3-one	Testosterone	T
4-androsten-3,17-dione	Androstenedione	A
4-androsten-17β-ol-3,11-dione	11-ketotestosterone	11-KT
4-pregnene-3,20-dione	progesterone	P
5-pregnen-3β-ol-20-one	pregnenolone	preg
4-pregnen-17α-ol-3,20-dione	17α-hydroxyprogesterone	17P
4-pregnene-17α,20β-diol-3-one	17α,20β-dihydroxy-4-pregnen-3-one	17,20βP
4-pregnene-17α,20β,21-triol-3-one	17α,20β,21-trihydroxy-4-pregnen-3-one	20βS
11β,17,21-trihydroxy-4-pregnene-3,20-dione	cortisol	F

abbreviations). Tubes were equilibrated for 30 min at room temperature and overnight at 4 °C. The incubation was terminated by addition of 0.5 ml DCC (10 $mg.ml^{-1}$). Tubes were vortexed and incubated at 4 °C for 5 min prior to centrifuging at 2060 g and 4 °C for 10 min. The supernatant was decanted, 5 ml of Ecolite scintillation cocktail (ICN Biochemicals) added, and vials were shaken and counted. An estimate of the dissociation constant (k_D) for each competing steroid, as a measure of binding affinity, was determined using the computer programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980).

Plasma samples were from fish from a variety of reproductive stages. Trout ranged from immature to mid-vitellogenic stage females. Flounder ranged from regressed to ovulated females, and snapper were either regressed or vitellogenic females. Bream were a mix of regressed males and females, and vitellogenic and final oocyte maturation stage females. The relative binding affinity of SBP for some steroids was investigated on pools of plasma that had not had endogenous steroids removed by pre-incubation with DCC. Due to a lack of sufficient previously unthawed plasma samples, the protocol was changed and the remaining assays were performed on plasma from individual fish. Endogenous steroids were removed to be consistent with the protocol for saturation assays (Table 4.2). Unlabelled E_2 was present as a competitor in every assay for consistency, so the binding of E_2 was analysed separately for pooled or DCC-stripped plasma. The 95 % confidence limits on the mean k_D calculated in LIGAND for each type of plasma overlapped for each species (Table 4.2). Therefore it was concluded that for the purposes of determining relative affinities of SBP for various ligands, there was no difference between values determined from pools of plasma containing endogenous steroids and plasma from individual fish with endogenous steroids removed. Accordingly, the k_D values reported in Tables 4.3 to 4.6 for E_2 are the combined results from assays with both types of plasma.

4.3 Results

Total binding was between 7.5 and 16.3 % of counts added for all species (Table 4.3). Specific binding was a high (greater than 66 % of total binding) in trout and bream, but less than 51 % of total binding in flounder and snapper (Table 4.3).

Table 4.2: Protocols and calculated values of k_D for plasma used to determine relative affinities of SBP for various steroids. Plasma for some assays was pooled and endogenous steroids were not stripped, while other assays were performed on DCC-stripped plasma from individual fish.

	Pool of plasma, not DCC-stripped		Individual fish, plasma DCC-stripped	
	T [1]		A	
	17,20βP [1]		P	
	F [1]		E_3	
	11-KT		preg	
	E_1		20βS	
	17P		E_2-3gluc	
			E_2-17gluc	
			E_2-3gluc-17sulf	
	k_D [2] (95 % CL) (nM)	n [2]	k_D [3] (95 % CL) (nM)	n [3]
trout	1.09 (0.63, 1.88)	4	0.64 (0.13, 3.12)	3
bream	4.41 (1.27, 15.26)	4	3.36 (0.92, 12.27)	4
snapper	11.1 (1.7, 73.1)	2	46.8 (0.2, 13127)	3
flounder	60.8 (20.1, 183.6)	4	73.7 (14.0, 388.5)	3

[1] Some plasma from flounder for these assays had been DCC-stripped.

[2] Assays using a pool of plasma, not DCC-stripped.

[3] Assays using DCC-stripped plasma from individual fish.

Table 4.3: Total binding, and specific binding as a percentage of total binding, for assays of relative binding affinity for trout, bream, snapper and flounder SBP. Values are mean ± se.

	^{3}H-E_2 (nM)	Total binding (% of counts added)	Specific binding (% of total)	n
Trout	5	16.3 ± 1.3	78.3 ± 2.5	10
Bream	5	7.9 ± 0.5	66.3 ± 1.6	10
Snapper	5	7.5 ± 0.8	42.6 ± 3.0	6
Flounder	5	9.2 ± 1.4	51.3 ± 6.3	7

E_2 bound to SBP with the highest affinity of all steroids tested in trout, and T had the second highest affinity for SBP (Fig. 4.1, Table 4.4). The relative affinity of T was about 30 % of E_2 in trout, with the relative affinity for all other steroids less than 5 % of that of E_2. Apart from E_2 and T, only 11-KT, A, E_3 and E_1 inhibited more than 70 % of bound E_2 at the highest concentrations of competing steroid. All other steroids tested with trout SBP competed for less than 44 % of specifically bound E_2, even at concentrations 10 000 times greater than E_2, and the maximum inhibition of binding with 17P, cortisol, 20βS and preg was less than 11 %. However, E_2-3gluc competed for about 60 % of E_2 specifically bound to trout SBP at the highest concentration tested (50 μM), although the relative affinity of binding of this steroid conjugate was still very low. There was no competition by the other E_2 conjugates. The maximum inhibition of binding differed between the 2 assays of trout SBP differed by less than 7 %.

Bream SBP displayed a marked preference for E_2, and all other steroids tested, including T, displayed relative affinities of less than 6 % of that of E_2 (Fig. 4.2, Table 4.5). There was greater than 69 % inhibition of binding of ^{3}H-E_2 to bream SBP by all unconjugated steroid competitors at the highest concentrations used. The E_2 conjugates did not compete with E_2 for SBP in bream. Unlike the case with trout, E_2-3gluc showed competition for less than 5 % of specifically bound steroid at a concentration of 50 μM. Differences in maximum inhibition of binding between assays for bream were less than 5 %.

E_2 was also bound with the highest affinity of all steroids investigated in snapper, and T bound with around 50 % of the affinity of E_2 (Fig. 4.3, Table 4.6). As for bream, the steroids with the next highest affinity were A, E_1 and 11-KT, however these steroids displayed less than 21 % of the affinity of E_2 for SBP. All competing steroids inhibited binding of more than 70 % of ^{3}H-E_2 to snapper SBP at the highest concentrations used, except for E_3 which only inhibited about 50 % of the bound E_2 at the highest concentration tested (50 μM). Generally the maximum inhibition of binding differed between the assays of snapper SBP by less than 10 %. The exception was for E_3 in snapper where the difference was around 21 %.

In flounder, T bound with the highest affinity, and affinity of E_2 was only about half that of T (Fig. 4.4, Table 4.7). The steroids with the next highest affinity were E_1, A and 11-KT,

Figure 4.1: Competition by various steroids for specific binding of ^{3}H-E_2 to trout SBP. Curves are representative of 2 assays.

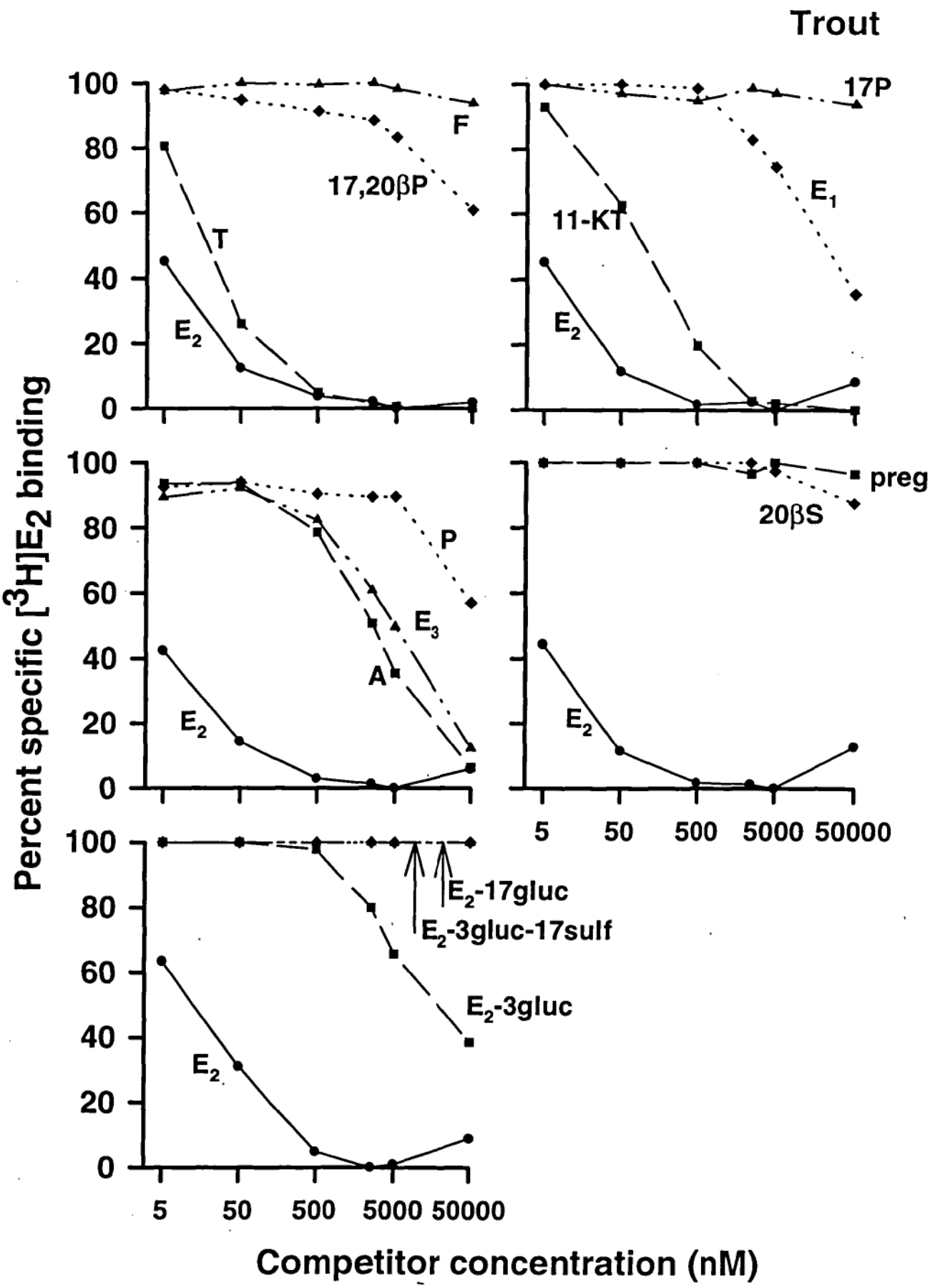

Trout
Percent specific [3H]E2 binding
100
80
60
40
20
0
F
17,20βP
T
E2
17P
E1
11-KT
E2
P
E3
A
E2
preg
20βS
E2
5
50
500
5000
50000
E2-17gluc
E2-3gluc-17sulf
E2-3gluc
E2
Competitor concentration (nM)

Table 4.4: Equilibrium dissociation constants (k_D) and relative affinity of various steroids for trout SBP.

Steroid	k_D (nM)	Maximum inhibition of specific binding (%)[1,2]	RA[3]
E_2	0.90 (n=7)	100	100
T	3.13	99.5	28.8
11-KT	20.9	100	4.3
A	357	94.4	0.3
E_3	631	85.9	0.1
E_1	1205[4]	70.7	0.07
E_2-3gluc	>4000[4]	60.9	0.02
P	>10000[4]	42.1	<0.01
17,20βP	>10000[4]	43.2	<0.01
17P	-	10.9	-
F	-	10.2	-
20βS	-	7.3	-
preg	-	1.8	-
E_2-17gluc	-	1.5	-
E_2-3gluc-17sulf	-	0.9	-

[1] Relative to maximum inhibition by E_2 adjusted to 100 %.

[2] Highest concentration was 50 μM for all steroids except for:
E_2 where the maximum inhibition was measured between 2.5 and 5 μM,
E_2-17gluc where the maximum inhibition of binding was measured at 2.5 μM
and E_2-3gluc-17sulf where maximum inhibition of binding was measured at 500 nM.

[3] Affinity relative to E_2 (%).

[4] Binding of E_2 was inhibited by 70 % or less at the highest concentrations of these competitors, thus k_D values should be viewed only as an indicator of affinity.

Figure 4.2: Competition by various steroids for specific binding of ^{3}H-E_2 to bream SBP. Curves are representative of 2 assays.

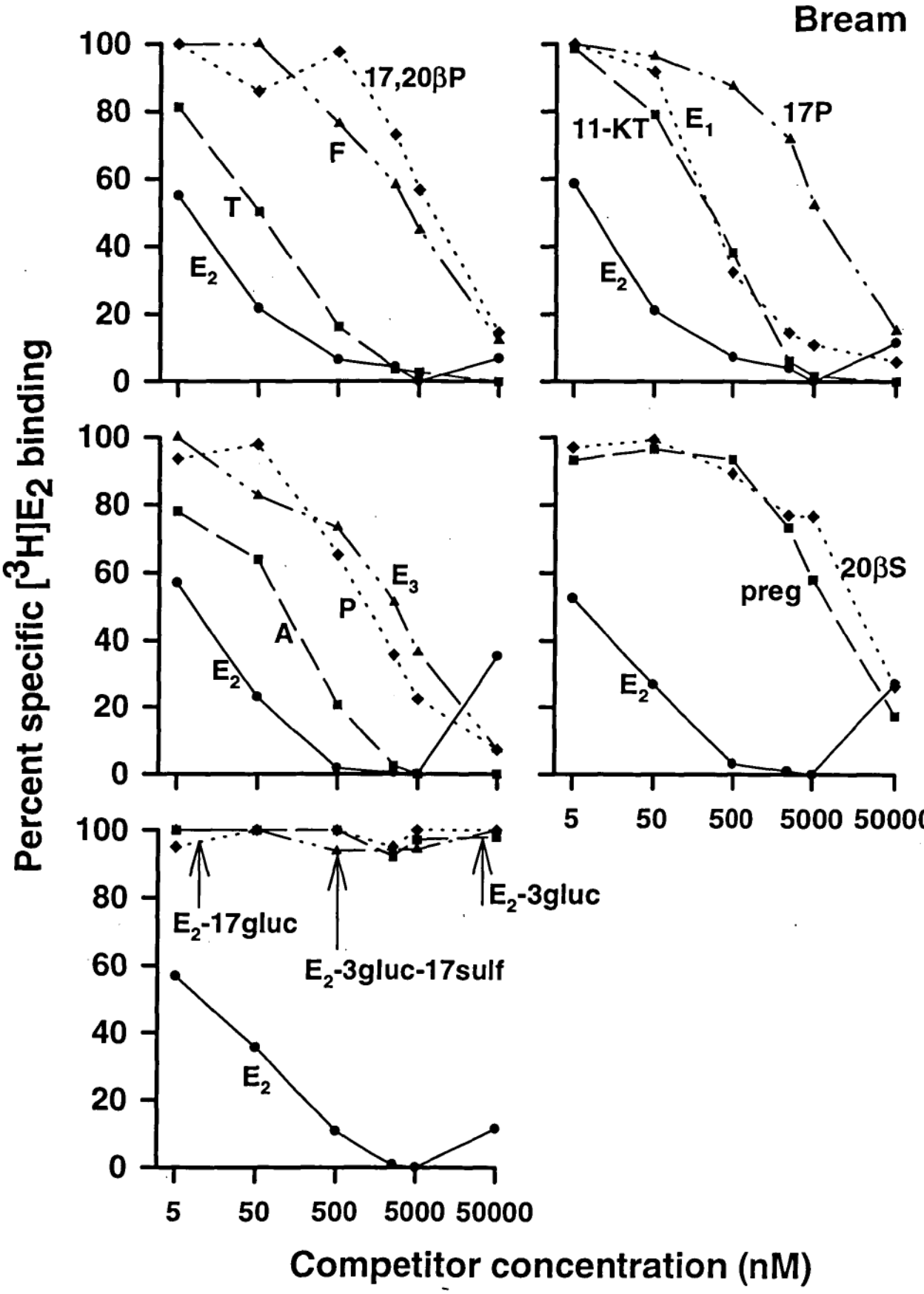

Bream
Percent specific [3H]E2 binding
Competitor concentration (nM)
17,20βP
F
T
E2
11-KT
E1
17P
E3
P
A
20βS
preg
E2-17gluc
E2-3gluc
E2-3gluc-17sulf
100
80
60
40
20
0
5
50
500
5000
50000

Table 4.5: Equilibrium dissociation constants (k_D) and relative affinity of various steroids for black bream SBP.

Steroid	k_D (nM)	Maximum inhibition of specific binding (%)[1,2]	RA[3]
E_2	3.91 (n=8)	100	100
T	64.9	100	6.0
A	91.2	100	4.3
E_1	95.9	93.7	4.1
11-KT	167	100	2.3
P	545	91.2	0.7
E_3	1340	90.1	0.3
17P	1956	84.1	0.2
F	1974	87.5	0.2
17,20βP	4243	85.5	0.1
Preg	4752	79.6	<0.1
20βS	>6000[4]	69.1	<0.1
E_2-3gluc-17sulf	-	11.1	-
E_2-3gluc	-	4.0	-
E_2-17gluc	-	2.5	-

[1] Relative to maximum inhibition by E_2 adjusted to 100 %.

[2] Highest concentration was 50 μM for all steroids except for:
E_2 and E_2-3gluc-17sulf where the maximum inhibition was measured at 5 μM,
and E_2-3gluc and E_2-17gluc where the maximum inhibition of binding was at 2.5 μM.

[3] Affinity relative to E_2 (%).

[4] Binding of E_2 was inhibited by 70 % or less at the highest concentrations of this competitor, thus k_D values should be viewed only as an indicator of affinity.

Figure 4.3: Competition by various steroids for specific binding of ^{3}H-E_2 to snapper SBP. Curves are representative of 2 assays.

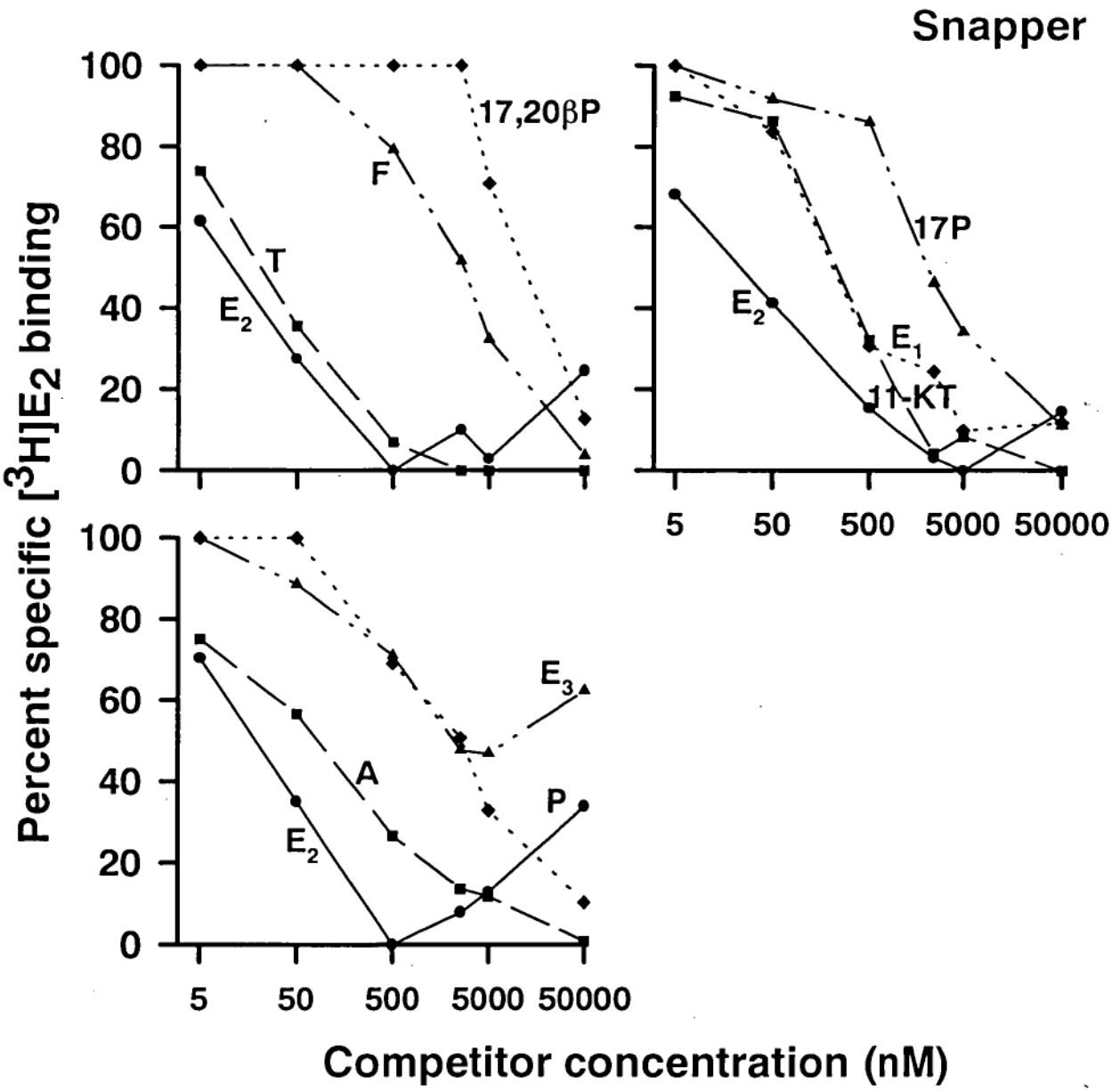

Snapper
Percent specific [3H]E2 binding
100
80
60
40
20
0
17,20βP
F
T
E2
17P
E1
11-KT
E3
A
P
5
50
500
5000
50000
Competitor concentration (nM)

Table 4.6: Equilibrium dissociation constants (k_D) and relative affinity of various steroids for snapper SBP.

Steroid	k_D (nM)	Maximum inhibition of specific binding (%)[1,2]	RA[3]
E_2	18.8 (n=5)	100	100
T	37.2	100	50.5
A	96.9	100	20.3
E_1	96.9	93.7	19.4
11-KT	318	100	5.9
F	1730	89.9	1.1
P	1892	80.8	1.0
17P	2328	84.6	0.8
E_3	>30000[4]	58.5	0.05
17,20βP	–	79.9	–

[1] Relative to maximum inhibition by E_2 adjusted to 100 %.

[2] Highest concentration was 50 μM for all steroids except for:
E_2 where the maximum inhibition was measured between 500 nM and 5 μM, and T where the maximum inhibition of binding was measured at 2.5 μM.

[3] Affinity relative to E_2 (%).

[4] Binding of E_2 was inhibited less than 70 % at the highest concentrations of this competitor, thus k_D values should be viewed only as an indicator of affinity.

Figure 4.4: Competition by various steroids for specific binding of ^{3}H-E_2 to flounder SBP. Curves are representative of 2 assays.

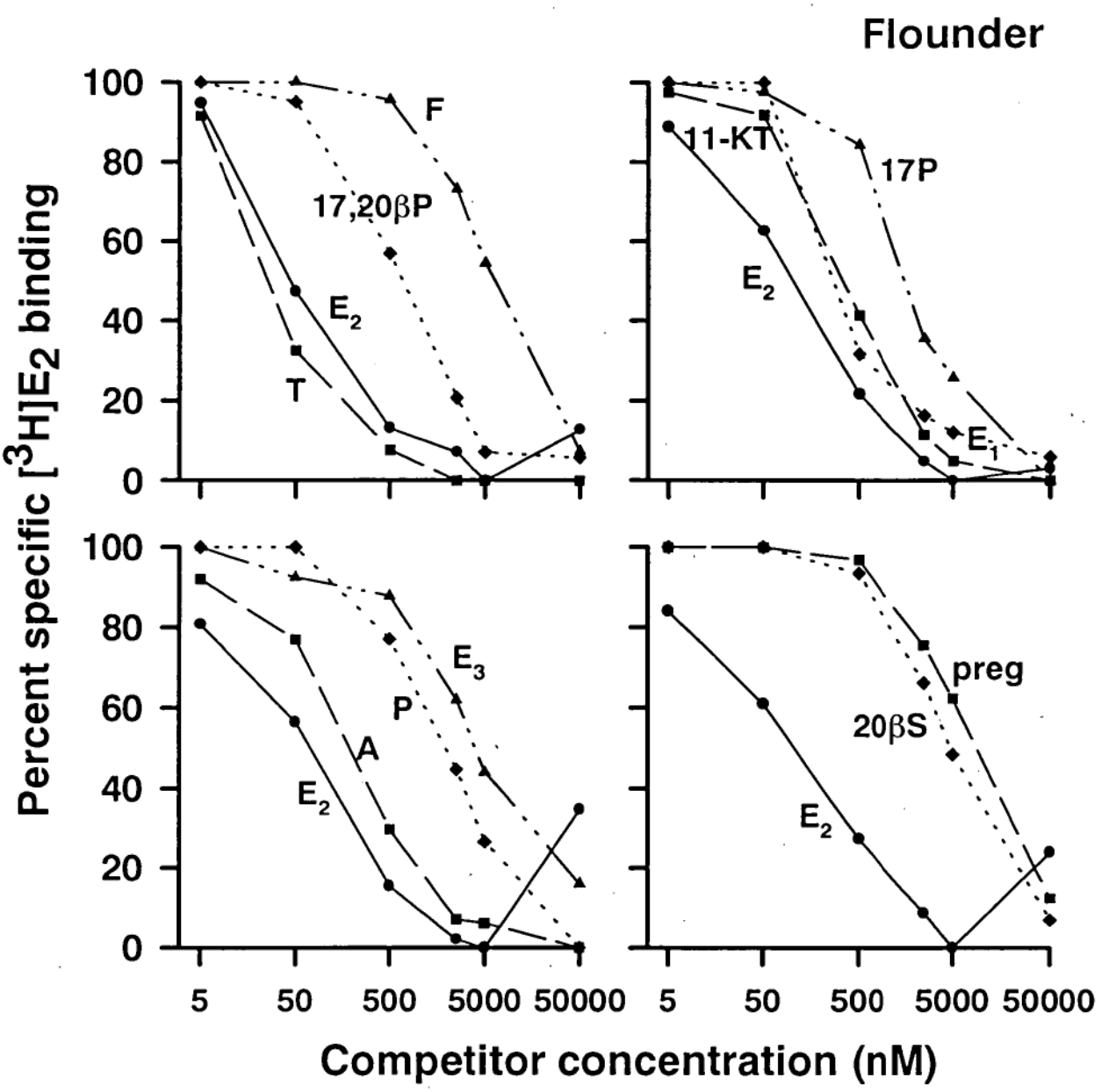

Flounder
Percent specific [3H]E2 binding
100
80
60
40
20
0
F
17,20βP
E2
T
11-KT
17P
E2
E1
E3
P
A
E2
preg
20βS
E2
5
50
500
5000
50000
Competitor concentration (nM)

Table 4.7: Equilibrium dissociation constants (k_D) and relative affinity of various steroids for greenback flounder SBP.

Steroid	k_D (nM)	Maximum inhibition of specific binding (%)[1,2]	RA[3]
T	35.6	100	215
E_2	76.6 (n=7)	100	100
E_1	220	94.7	34.8
A	368	100	20.8
11-KT	382	100	20.1
17,20βP	740	94.9	10.3
P	1372	94.4	5.6
17P	1872	97.4	4.1
20βS	2982	98.9	2.6
E_3	5560[4]	71.5	1.4
F	5643	84.8	1.4
Preg	7884	90.4	0.1

[1] Relative to maximum inhibition by E_2 adjusted to 100 %.

[2] Highest concentration was 50 μM for all steroids except for:
E_2 where the maximum inhibition of binding was measured between 2.5 and 5 μM.

[3] Affinity relative to E_2 (%).

[4] Binding of E_2 was inhibited by around 70 % at the highest concentrations of this competitor, thus k_D values should be viewed only as an indicator of affinity.

however, these steroids were bound with less than 35 % of the affinity of E_2. There was greater than 71 % inhibition of binding of ^{3}H-E_2 to flounder SBP by all steroid competitors at the highest concentrations tested. The maximum inhibition of binding differed between the assays of flounder SBP by less than 10 %.

The calculated values for k_D from the 2 assays for each steroid varied more than the maximum inhibition of binding. For steroids with k_D values of less than 1μM, differences between the two assays of trout and bream plasma were 7 to 16 % and 6 to 32 % respectively. Differences ranged from 1 to 71 % for flounder assays, and 13 to 41 % for snapper assays of steroids with k_Ds below 1 μM. However, even when the difference between k_D measured from the 2 assays was greater than 20 %, the rank order of affinity remained the same, and the affinities relative to that of E_2 were still of the same order of magnitude.

4.4 Discussion

The binding proteins under investigation had variable specificity for different steroids in different species. The proteins primarily bind E_2 and T with high affinity, but other steroids are also bound with lower affinity providing further support that the plasma proteins in these four species are SBPs. Similarly, in all species estrogens and androgens were more effective competitors of ^{3}H-E_2 binding than progestogens and cortisol. This supports the developing consensus that separate binding systems for E_2/T and progestogens/ corticosteroids operate in teleosts (Fostier and Breton, 1975; Caldwell *et al.*, 1991).

Total binding was low (<17 %) for all species, satisfying the assay requirement that the amount of binding protein be as small as possible such that the amount of ligand added approximates the concentration of free radioligand, and effects of affinity measurements are minimised (Kenakin, 1993). Specific binding to trout and bream SBP was sufficiently high (78.3 and 66.3 % respectively), that the difference in k_D values measured for the 2 assays was generally less than 10 %. However, as k_D increased, there was greater variation between the assays (up to 50 % difference between assays of steroids with $k_D > 1$ μM for trout and bream). This is to be expected as the higher k_D indicates less specific binding to

the SBP. Specific binding to flounder and snapper SBP, however, was lower (51.3 and 42.6 % respectively). As a consequence of the lower specific binding, there was higher variation between the assays (13 - 41 and 1 - 71 % for steroids binding with k_Ds below 1 μM to snapper and flounder SBP respectively). For these 2 species, the k_D values measured must be viewed with caution, however, the rank order of affinity of the steroids is the same or similar to that for trout and bream SBP.

Even though E_2 was one of the 2 most effective competitors in all species, at the highest concentrations of unlabelled E_2 used in the assays, inhibition of binding was incomplete. This rise in specifically bound ^{3}H-E_2 in most assays at the highest unlabelled E_2 concentration appears to be an artefact, probably resulting from the low solubility of steroids at high concentrations in the aqueous assay buffer.

E_2 was the most effective competitor for ^{3}H-E_2 binding in trout. The relative affinity of SBP for T compared to E_2 in trout was about 30 % and the relative affinity of all other steroids was less than 5 % of that of E_2. The rank order of affinity of binding of steroids to trout SBP (E_2 > T > 11-KT > A > E_3 > E_1) agrees fairly well with data from other salmonids including female rainbow trout (Pottinger and Pickering, 1990) and female brown trout *Salmo trutta* (Pottinger, 1986). In several other studies on salmonids, however, T has been reported to have higher affinity than E_2. SBP produced in hepatocyte culture from rainbow trout, and plasma SBP from brown trout both bound T with higher affinity than E_2 (Pottinger, 1987; Foucher *et al.*, 1991). In both studies where this was the case, the fish used were males and the radiolabelled ligand was T. It is possible that the difference in relative binding of E_2 and T may be a sex difference. However, in an early study on rainbow trout SBP, Fostier and Breton (1975) investigated binding of various steroids using both ^{3}H-E_2 and ^{3}H-T. In both cases T was bound with higher affinity than E_2 in these female fish, although the difference in relative affinities was less when the radiolabelled ligand was E_2. This suggests that the relative binding affinities differ depending on whether T or E_2 is used as the radiolabelled ligand. Other studies also confirm the greater displacement of a tracer with a homologous rather than heterologous competing ligand. In a study on the green frog *Rana esculenta*, E_2 bound in preference to T in assays with ^{3}H-E_2, and T was more effective at displacing ^{3}H-T for both male and female frogs (Paolucci and Di Fiore, 1994), also suggesting a sex difference is unlikely. E_2

was bound with similar relative affinity as T to SBP in 4 amphibian species in assays using ^{3}H-E_2. However, when ^{3}H-T was used, the relative affinity for T was consistently higher (Martin and Ozon, 1975). A similar pattern is seen in a study with rainbow trout (Milligan *et al.*, 1998), but not with goldfish *Carassius auratus* (Pasmanik and Callard, 1986) where T is reported as the ligand with higher affinity from both ^{3}H-E_2 and ^{3}H-T assays. However, data in the results of the study by Pasmanik and Callard (1986) suggest that the affinity of E_2 is the same as that of T when ^{3}H-E_2 is used as the radiolabelled ligand, and lower than T when ^{3}H-T is the labelled ligand, in disagreement with the text (Pasmanik and Callard, 1986). It is possible that T may also be more effective at displacing ^{3}H-T from SBP in the rainbow trout, bream and snapper used in our study, but this possibility was not investigated.

In bream, E_2 was considerably more effective as a competitor than T. The affinity of T was only 6 % of that of E_2. This suggests that bream SBP acts more as an estradiol binding protein, as the concentrations of E_2 and T in plasma remain similar to each other throughout the seasonal and diurnal cycles (Haddy and Pankhurst, 1998), and hence the concentration of T may not be sufficient at any stage to displace a measurable amount of E_2. In snapper, T was bound with 50 % and A and E_1 with about 20 % of the affinity of E_2. The rank order of affinity of the steroids competing for ^{3}H-E_2 was the same in snapper and bream for the 5 steroids with highest affinity (E_2 > T > A > E_1 > 11-KT). However, snapper SBP bound E_2 with lower affinity and T with higher affinity than bream SBP. The difference in relative affinity for T between the two sparid species is unexpected, and may be an artefact of the different treatment of the plasma before assay. Bream plasma used to determine relative affinities for steroids was thawed only for these assays and not been thawed previously. Snapper plasma, however, was sourced from another study and had been transported thawed before use in the present study. No significant changes of affinity in trout, bream or flounder SBPs with freezing and storage were detected (Chapter 2, this volume), but this was not investigated with snapper plasma as no fresh plasma was available for assay during the present study.

In contrast to the results from the other species, the relative binding affinity of T to flounder SBP was more than twice that of E_2, therefore in this case there is certainty that T is the more effective ligand. Flounder SBP also showed lower specificity, with the steroids

E_1, A and 11-KT binding with relative affinities of more than 20 % of E_2. This lower specificity is in contrast to the results for trout and bream, but similar to results obtained with snapper SBP. Initially the lower affinity measured for binding of E_2 to flounder SBP suggested the possibility of a different type of protein, but the relative order of binding of estrogens, androgens, progestogens and cortisol has confirmed that E_2 is binding to SBP and not, for example, to a CBG-like protein.

The rank order of affinity of steroids binding to SBP in all 4 species (E_2 or T, followed by other androgens and estrogens (with the exception of E_3) which have moderate affinities, then progestogens and cortisol with low affinities) is similar to that in the salmonids as mentioned previously, and also to the few non-salmonids studied, including goldfish (Pasmanik and Callard, 1986; Van Der Kraak and Biddiscombe, 1999), carp *Cyprinus carpio* (Chang and Lee, 1992) and spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1994). The relative affinity of SBP for different steroids is related to the steroid structure. For a steroid to bind with high relative affinity to human SBP, the requirements are for a planar steroid nucleus, an unhindered 17β-hydroxyl group and preferably a carbonyl group at C-3 (reviewed in Lobl, 1981; Petra, 1991), hence the high affinity of human SBP for T and DHT. The affinity of human SBP for E_2 is slightly lower reflecting that binding is stronger with a ketone than a hydroxyl at C-3. Similar chemical criteria appear to exist for binding to teleost SBP. The steroids with highest affinity binding to trout, bream, snapper and flounder SBP were E_2 and T, both of which have an unhindered 17β-hydroxyl group and possess either a 3-hydroxyl (E_2) or a 3-ketone group (T). Oxidation of the 17β-hydroxyl group to a carbonyl group (A compared to T; E_1 compared to E_2) reduced the binding affinity, and addition of other groups to C-17 (eg. progestogens) removed any ability of the steroids to bind to SBP at physiological concentrations. Several conjugated forms of E_2 were also investigated to confirm the importance of the functional groups attached to C-3 and C-17. The presence of a glucuronide group on C-3 or C-17 completely removed any ability of the steroid to bind to bream SBP. However, binding to trout SBP was still observed with E_2 bearing a glucuronide at C-3, but the presence of a glucuronide on C-17 prevented any binding in trout. This suggests that for trout SBP at least, the binding affinity is dictated firstly by the functional group on C-17, and then subsequently by any groups attached to C-3. The relative affinity of E_1 compared to E_2

ranged from ~35 % (flounder) down to less than 0.1 % (trout). This reduction in affinity results from removal of a single hydrogen from the oxygen attached to C-17, suggesting the 17β-hydroxyl group is required more for its ability to form bonds with the appropriate amino acid in the SBP binding site, than because of space constraints in the binding domain.

All of the progestogens investigated in the present study differ from androgens and estrogens by the attachment of a carbon side chain from the β-face of C-17. Therefore, it is difficult to determine whether the reduction in affinity resulted from a lack of space for the extra side chain within the binding site, or removal of the 17β-hydroxyl. The complete lack of binding of E_2 with a glucuronide group conjugated to C-17, however, suggests that both the presence of the 17β-hydroxyl and the size of the group around C-17 are important. The glucuronide group attached to the α-face of C-17 probably interferes with binding of the 17β-hydroxyl group to SBP by altering the fit of the steroid in the binding site. It has been hypothesised that human SBP binds strongly to the β-face of the steroids and to the C-3 and C-17 groups, and that edge changes to the basic E_2 / T structure also reduce binding affinity (Lobl, 1981). The results from the present study suggest that the SBP molecule has been fairly well conserved through evolution, and the structure-affinity relationships that hold for mammals are also similar for the teleosts.

4.5 References

Caldwell, C.A., Kattesh, H.G., and Strange, R. (1991). Distribution of cortisol among its free and protein-bound fractions in rainbow trout (*Oncorhynchus mykiss*): evidence of control by sexual maturation. *Comp. Biochem. Physiol.* **99A**, 593-595.

Callard, I.P., and Callard, G.V. (1987). Sex steroid receptors and non-receptor binding proteins. *In* "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D.O. Norris, and R.E. Jones, Eds.), pp. 355-384. Plenum Press, New York.

Chang, C.F., and Lee, Y.H. (1992). Purification of the sex steroid-binding protein from common carp (*Cyprinus carpio*) plasma. *Comp. Biochem. Physiol.* **101B**, 587-590.

Fostier, A., and Breton, B. (1975). Binding of steroids by plasma of a teleost: the rainbow trout, *Salmo gairdnerii*. *J. Steroid Biochem.* **6**, 345-351.

Foucher, J.L., Niu, P.D., Mourot, B., Vaillant, C., and Le Gac, F. (1991). *In vivo* and *in vitro* studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Molec. Biol.* **39**, 975-986.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Hammond, G.L. (1990). Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocr. Rev.* **11**, 65-79.

Joseph, D.R. (1994). Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197-280.

Kenakin, T.P. (1993). Radioligand binding experiments. *In* "Pharmacologic Analysis of Drug-Receptor Interaction" 2nd edn, pp. 385-410. Raven Press, New York.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Lobl, T.J. (1981). Androgen transport proteins: physical properties, hormonal regulation, and possible mechanism of TeBG and ABP action. *Arch. Androl.* **7**, 133-151.

Martin, B. (1975). Steroid-protein interactions in nonmammalian vertebrates. I. Elasmobranch steroids-binding protein (E.SBP) in dogfish serum (*Scyliorhinus canicula*). *Gen. Comp. Endocrinol.* **25**, 42-51.

Martin, B., and Ozon, R. (1975). Steroid-protein interactions in nonmammalian vertebrates. II. Steroids binding proteins in the serum of amphibians; a physiological approach. *Biol. Reprod.* **13**, 371-380.

McPherson, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacological Methods* **14**, 213-228.

Milligan, S.R., Khan, O., and Nash, M. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (*Oncorhynchus mykiss*) plasma. *Gen. Comp. Endocrinol.* **112**, 89-95.

Munson, P.J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.

Nelson, J.S. (1984). "Fishes of the World", p. 523. John Wiley & Sons, New York.

Paolucci, M., and Di Fiore, M.M. (1994). Sex steroid binding proteins in the plasma of the green frog, *Rana esculenta*: Changes during the reproductive cycle and dependence on pituitary gland and gonads. *Gen. Comp. Endocrinol.* **96**, 401-411.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Molec. Biol.* **40**, 735-753.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1987). Androgen binding in the skin of mature male brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **66**, 224-232.

Pottinger, T.G., and Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **80**, 264-273.

Renoir, J.M., Mercier-Bodard, C., and Baulieu, E.E. (1980). Hormonal and immunological aspects of the phylogeny of sex steroid binding plasma protein. *Proc. Natl. Acad. Sci. USA* **77**, 4578-4582.

Salhanick, A.C.R., and Callard, I.P. (1980). A sex-steroid-binding protein in the plasma of the freshwater turtle, *Chrysemys picta*. *Gen. Comp. Endocrinol.* **42**, 163-166.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

5. Differences in binding characteristics of sex steroid binding protein (SBP) between reproductive and non-reproductive female rainbow trout (*Oncorhynchus mykiss*), black bream (*Acanthopagrus butcheri*) and greenback flounder (*Rhombosolea tapirina*)

5. Differences in binding characteristics of sex steroid binding protein (SBP) between reproductive and non-reproductive female rainbow trout (*Oncorhynchus mykiss*), black bream (*Acanthopagrus butcheri*) and greenback flounder (*Rhombosolea tapirina*)

5.1 Introduction

Estradiol (E_2) and testosterone (T) are transported in the plasma of many species bound to a sex steroid binding protein (SBP). Binding to SBP protects steroids from metabolism - the metabolic clearance rate of T, for example, is reduced in the presence of SBP (Petra *et al.*, 1983). As well as protecting steroids in the plasma, SBPs may also act to control steroid entry to target cells (Petra *et al.*, 1983; Stanczyk *et al.*, 1986; Petra, 1991). Specific cell surface receptors have been found for the protein in target areas, and although only unliganded SBP can bind to the receptor, steroid can then bind to the SBP-receptor complex (Hryb *et al.*, 1986, 1990). SBP has also been found inside cells (Bordin and Petra, 1980; Porto *et al.*, 1992), although it is not certain whether this SBP is of extra- or intracellular origin (Baulieu, 1986).

Sex steroid levels vary throughout the reproductive cycle in all vertebrates (Norris, 1997). In female fish, plasma levels of E_2 and T tend to peak with vitellogenesis, whereas levels of maturation-inducing steroids (usually progestogens) are elevated during final oocyte maturation and ovulation (reviewed in Pankhurst, 1998). Peak steroid levels vary widely between species. Female salmonids, for example, have levels of T and E_2 of about 140 to 200 $ng.ml^{-1}$ and 30 to 50 $ng.ml^{-1}$ respectively (Scott *et al.*, 1980; Pankhurst and Thomas, 1998), whereas many marine species and repeat spawning species seldom have levels of E_2 and T above 10 $ng.ml^{-1}$ (Pankhurst and Carragher, 1991; Carragher and Pankhurst, 1993; Haddy and Pankhurst, 1998).

The relationship between plasma levels of SBP (binding capacity) and plasma levels of sex steroids (reproductive stage) has been investigated in a limited number of non-mammalian vertebrates. SBP levels are highest when plasma concentrations of E_2 are highest in female

spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1997) and the female green tree frog *Rana esculenta* (Paolucci and Di Fiore, 1994). The opposite pattern is seen with T in male brown trout *Salmo trutta*, where binding capacity is lowest when T levels are highest (Pottinger, 1988), and the same phenomenon may be present in male rainbow trout *Oncorhynchus mykiss* (Foucher *et al.* 1992). Female alligators *Alligator mississippiensis*, display the lowest SBP levels during breeding, with minimum SBP levels at the same time as the rapid decline in plasma E_2 (Ho *et al.*, 1987). In contrast, no differences in SBP levels in relation to reproductive stage were found in female goldfish *Carassius auratus* (Pasmanik and Callard, 1986), male or female carp *Cyprinus carpio* (Chang and Chen, 1990, 1991) or male newts *Taricha granulosa* (Moore *et al.*, 1983).

The aim of the present study was to compare plasma SBP levels in vitellogenic fish (the stage with the highest levels of E_2 and T) with SBP concentrations in non-reproductive fish (where lowest E_2 and T levels typically occur). These differences were investigated in three species of teleosts which have different peak plasma levels of steroids, and SBPs with different binding capacity and affinity for ligands (Chapter 3, this volume). Rainbow trout (*O. mykiss*) have peak levels of E_2 and T of about 50 and 200 $ng.ml^{-1}$ respectively (Scott *et al.*, 1980) and a very high affinity (0.44 nM) SBP with moderate capacity (92 nM) (Chapter 3, this volume). In contrast, black bream (*Acanthopagrus butcheri*, Sparidae) are multiple repeat spawners, probably on a daily basis, with peak E_2 and T levels of 4.2 and 2.9 $ng.ml^{-1}$ respectively (Haddy and Pankhurst, 1998). Black bream also have a high affinity SBP, but the affinity (3.39 nM) and capacity (50 nM) are both lower than in rainbow trout (Chapter 3, this volume). Greenback flounder (*Rhombosolea tapirina*, Pleuronectidae) are also multiple repeat spawners, over a prolonged spawning season, but ovulatory periodicity is unknown (Barnett and Pankhurst, 1999). Peak plasma levels of E_2 and T in greenback flounder are slightly higher than in black bream at 3 $ng.ml^{-1}$ and 8 $ng.ml^{-1}$ for E_2 and T respectively (Barnett and Pankhurst, 1999), but the SBP has markedly lower affinity for E_2 and T (k_D = 85 nM), along with higher capacity (164 nM) than either trout or bream SBP (Chapter 3, this volume).

In addition to changes in binding capacity, SBP may also show changing affinity with reproductive status. Changes in k_D with reproductive stage have been reported for female spotted seatrout, with higher k_D (lower affinity) associated with higher plasma steroid

levels (Laidley and Thomas, 1997). In the present study, the relative affinity of SBP for 4 ligands was assessed in fish of different reproductive stages to determine whether there were changes in k_D for E_2 with reproductive stage, and if so, whether the relative affinity for various steroids also changed (possibly indicating another form of the binding protein).

5.2 Methods

Plasma Collection

Greenback flounder used in the present study were collected as described in Barnett (1998). Black bream were sampled from the wild as described in Haddy and Pankhurst (1998). Samples of rainbow trout plasma were collected from fish maintained at the Aquatic Centre of the University of Tasmania as described in Thomas *et al.* (1999). The reproductive characteristics of the fish are reported in Table 5.1.

Blood samples were taken from female fish by caudal puncture with heparinised 22G needles. Blood samples were centrifuged and the plasma removed and stored at -20 °C. Before assay, plasma was stripped of endogenous steroids by incubation with an equal volume of 20 $mg.ml^{-1}$ dextran-coated charcoal (DCC) for 20 min, with vortexing immediately and 10 min after addition of DCC, followed by centrifugation for 10 min. The supernatant was decanted and diluted (50-fold for flounder, 100-fold for bream and 200-fold for trout) for use in assay.

Binding Assay

The charcoal adsorption binding assay has been previously described (Hobby and Pankhurst, 1997; Chapter 2, this volume). In brief, DCC-stripped plasma was diluted in phosphate buffer and incubated with a range of labelled E_2 ([2,4,6,7-^{3}H]estradiol (Amersham)) concentrations (0.25 - 20 nM for trout and 1 - 20 nM for bream) both with and without 100-fold excess unlabelled E_2 as competitor. For estimation of k_D and B_{max} in flounder SBP, a cold saturation protocol was used. DCC-stripped, 50-fold diluted flounder plasma was incubated with 5 nM ^{3}H-E_2 and increasing concentrations (5 - 2500 nM) of unlabelled E_2. Tubes were equilibrated 30 min at room temperature and overnight at 4 °C. The incubation was terminated by addition of 0.5 ml 10 $mg.ml^{-1}$ DCC. Tubes were

Table 5.1: Reproductive characteristics for non-reproductive and vitellogenic female trout, bream and flounder.

	Non-reproductive			Vitellogenic [1]		
	E_2 ($ng.ml^{-1}$) (mean ± se)	T ($ng.ml^{-1}$) (mean ± se)	GSI (%)	E_2 ($ng.ml^{-1}$) (mean ± se)	T ($ng.ml^{-1}$) (mean ± se)	GSI (%)
trout	-	-	- [2]	18.9 ± 1.2	9.5 ± 0.7	-
bream [3]	0.25 ± 0.04	0.18 ± 0.00	1.0 ± 0.1	1.25 ± 0.17	0.36 ± 0.10	5.5 ± 0.3
flounder	<1 [4]	<1 [4]	4.3 ± 0.3 [4]	>3 [5]	>3 [5]	14.3 ± 1.0

[1] Confirmed by macroscopic observation.

[2] Measurements were not obtained. Gonads ranged from indistinguishable (immature) to regressed.

[3] Measurements are a subset of data collected by Haddy and Pankhurst (1998).

[4] Animals were sampled outside of the spawning season (Barnett, 1998) and returned to the tanks for subsequent experiments. Values reported are from spent females collected by Barnett and Pankhurst (1999).

[5] Values from vitellogenic female fish from a previous study (Barnett and Pankhurst, 1999).

vortexed and incubated at 4 °C for 5 min prior to centrifuging at 3000 rpm and 4 °C for 10 min. The supernatant was decanted, 5 ml of Ecolite scintillation cocktail added and vials were shaken and counted using a standard tritium procedure. Specific binding was calculated by subtracting non-specific binding from total binding. The dissociation constant (k_D), maximum binding capacity (B_{max}) and 95 % confidence limits on these parameters for E_2 were calculated using the binding analysis programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980).

Competitive Binding Assay

The relative affinity of SBP for E_2, T, 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and cortisol was assessed in fish of different reproductive stages. The relative affinity of T was measured as the steroid with the next highest affinity after E_2 in trout and bream, and the steroid with highest affinity in flounder. 17,20βP is the maturation inducing steroid (MIS) in trout (reviewed by Scott and Canario, 1987), and may also be the MIS in sparids (e.g. Ventling and Pankhurst, 1995) and greenback flounder (Barnett, 1998). There is evidence for differential effects of stress/cortisol on reproductive versus non-reproductive fish (Pankhurst and Van Der Kraak, 1997). In view of the stress/cortisol-induced decrease in B_{max} of SBP (Hobby *et al.*, 2000; Chapter 6, this volume), it was necessary to determine whether the affinity of SBP for cortisol might differ between fish of different reproductive stages. E_2 was included in each assay to allow comparisons between assays. DCC-stripped plasma was diluted in phosphate buffer and incubated with ^{3}H-E_2 at a final concentration of 5 nM, and increasing concentrations (0, 5, 50, 500 nM, 2.5, 5, 50 μM) of unlabelled competitor. Tubes were then incubated and processed as above. Estimates of the k_D for T, 17,20βP and cortisol were determined using the computer programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980). These were then compared to the k_D calculated for E_2 to give an estimate of the relative binding affinity.

Trout E_2 Injection Experiment

To determine whether a measured difference in binding capacity between vitellogenic and non-reproductive female trout was a result of high plasma E_2 or vitellogenin levels, a group of male rainbow trout were injected with E_2 to stimulate production of vitellogenin, thus

simulating late vitellogenic stage female fish. Ten fish were netted, anaesthetised, weighed, tagged, and a blood sample was taken from the caudal vessel. Five fish were injected intraperitoneally with E_2 in saline at a dose of 5 $mg.kg^{-1}$ body weight. The remaining 5 fish were injected with the same volume of saline, as controls. The fish were fed 3 times a week and maintained as described by Thomas *et al.* (1999). The fish were injected twice more at weekly intervals, and a final blood sample was taken 1 week after the 3rd injection. The plasma was removed and stored at -20 °C until assay. The SBP in plasma samples was partially purified by gel filtration to remove most of the vitellogenin before reassay to determine if measured changes in SBP binding characteristics resulted from high plasma vitellogenin levels. An equal volume of plasma and Tris buffer (0.05 M adjusted to pH 8 with 1 M HCl) were mixed, and 2 ml of the diluted sample was applied to a column (1.8 x 84 cm) of Sephacryl S-300-HR (Sigma). The sample was eluted with 0.05 M Tris at a flow rate of approximately 9 $ml.h^{-1}$ and fractions were collected every 13.5 min. The procedure was carried out at 4 °C and absorbance of the fractions was measured at 280 nm. The approximate molecular weight of trout SBP was determined by use of molecular weight markers (MW-GF-1000 kit, Sigma). Fractions containing SBP binding activity were identified by incubating duplicate tubes of 50 µl of each fraction with 50 µl of ^3H-E_2 (final concentration 10 nM) and 150 µl phosphate buffer. The remainder of the assay protocol was as described above. To determine binding affinity and capacity in the E_2-binding fractions identified after gel filtration, fractions containing 50 % of the binding to E_2 were pooled, and stripped 3 times with DCC to remove any remaining steroids. The E_2-binding fractions used for assay were at a dilution equivalent to the 200-fold dilution used for plasma. Determination of k_D and B_{max} was as described above for trout.

Statistics

Significant differences were detected when 95 % confidence limits on k_D or B_{max} estimates calculated in LIGAND (Munson and Rodbard, 1980) did not overlap.

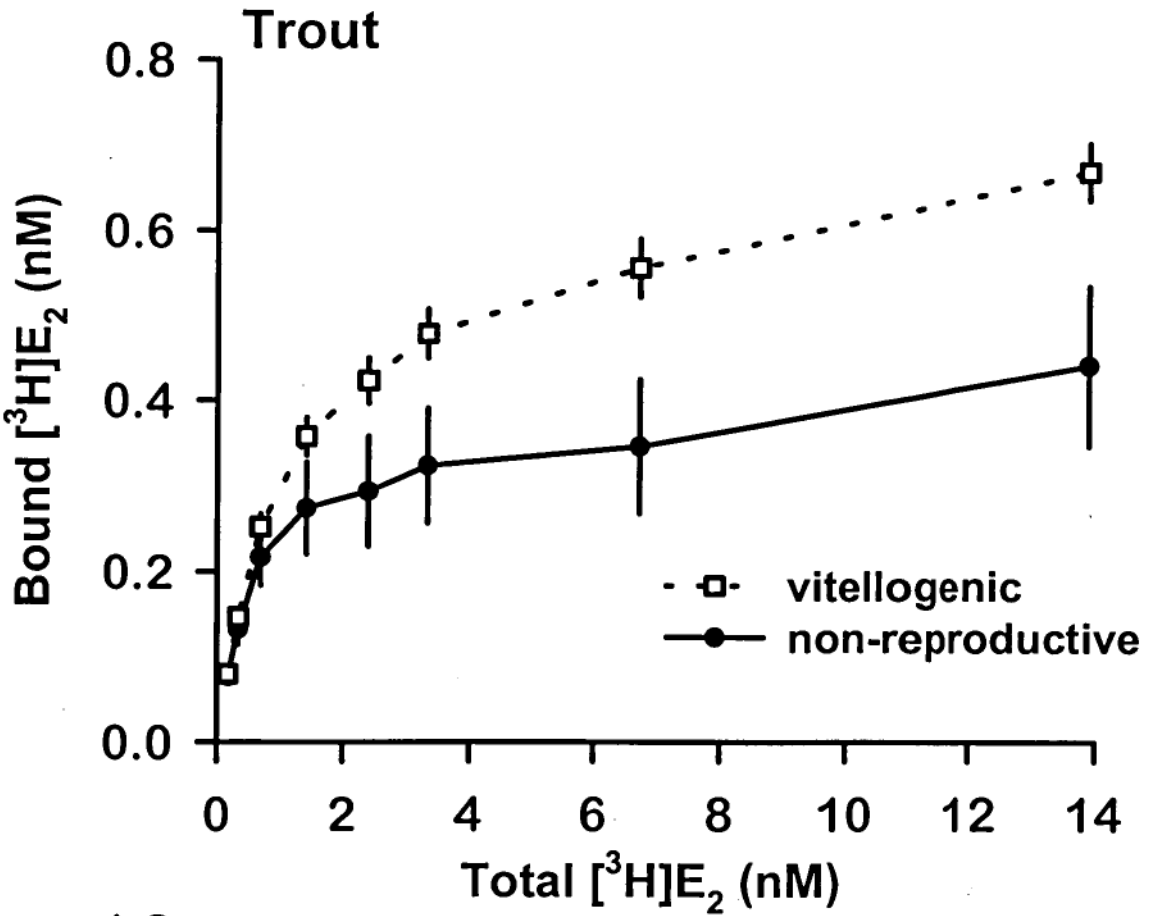
5.3 Results

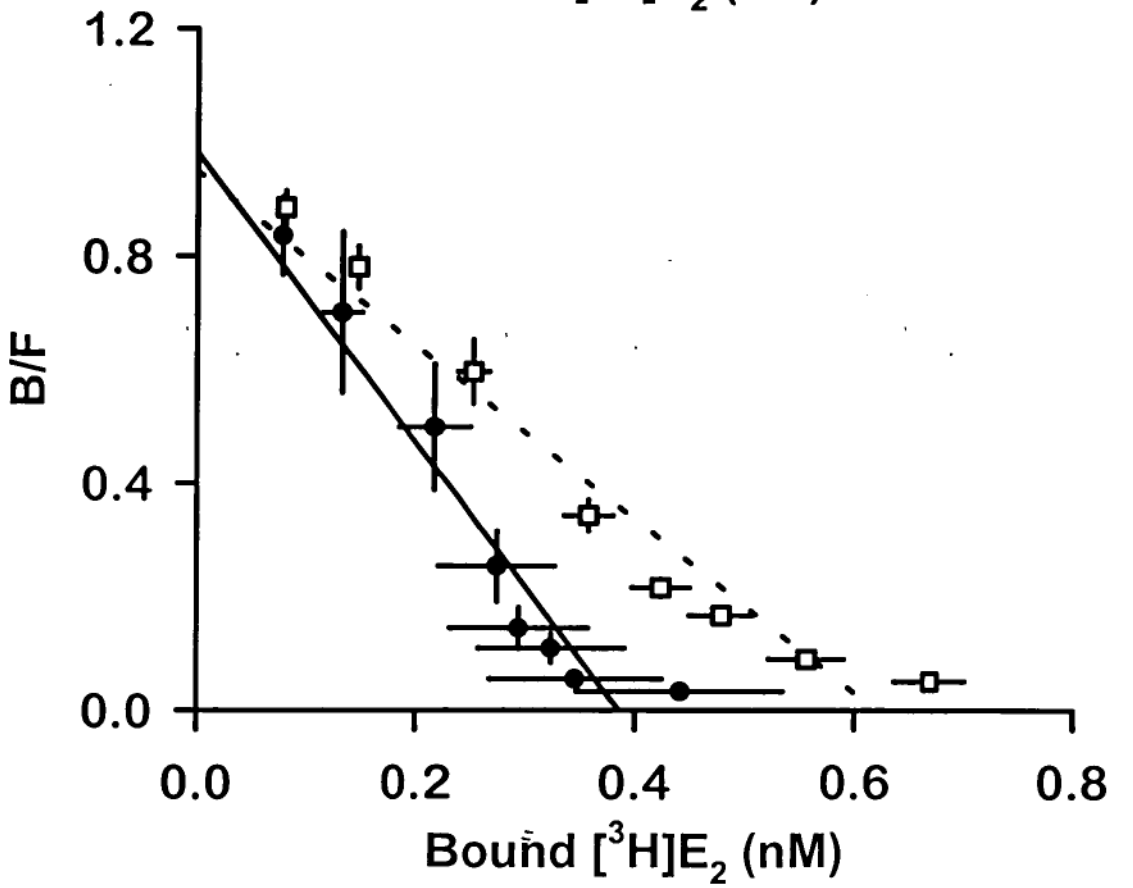
The binding capacity of trout plasma was significantly greater in vitellogenic fish (124 nM) than in non-reproductive fish (77 nM) (Table 5.2, Fig. 5.1). Binding capacity of SBP in

Table 5.2: Mean binding capacity for non-reproductive and vitellogenic trout, bream and flounder, with upper and lower 95 % confidence limits calculated from estimates produced by LIGAND. Significant differences (non-overlapping confidence limits) between non-reproductive and vitellogenic fish are shown by *.

	Non-reproductive			Vitellogenic	
Species	B_{max} (nM)	(95 % CL)		B_{max} (nM)	(95 % CL)
trout	77	(63, 94)	*	124	(115, 134)
bream	39	(33, 46)	*	60	(50, 71)
flounder	407	(283, 585)		398	(268, 589)

Figure 5.1: Saturation curve (upper) and Scatchard (lower) plots for SBP in plasma of vitellogenic and non-reproductive trout. Points are mean ± se, n = 5 fish.


Trout
Bound [^{3}H]E$_2$ (nM)
0.0
0.2
0.4
0.6
0.8
0
2
4
6
8
10
12
14
Total [^{3}H]E$_2$ (nM)
vitellogenic
non-reproductive


B/F
0.0
0.4
0.8
1.2
0.0
0.2
0.4
0.6
0.8
Bound [^{3}H]E$_2$ (nM)

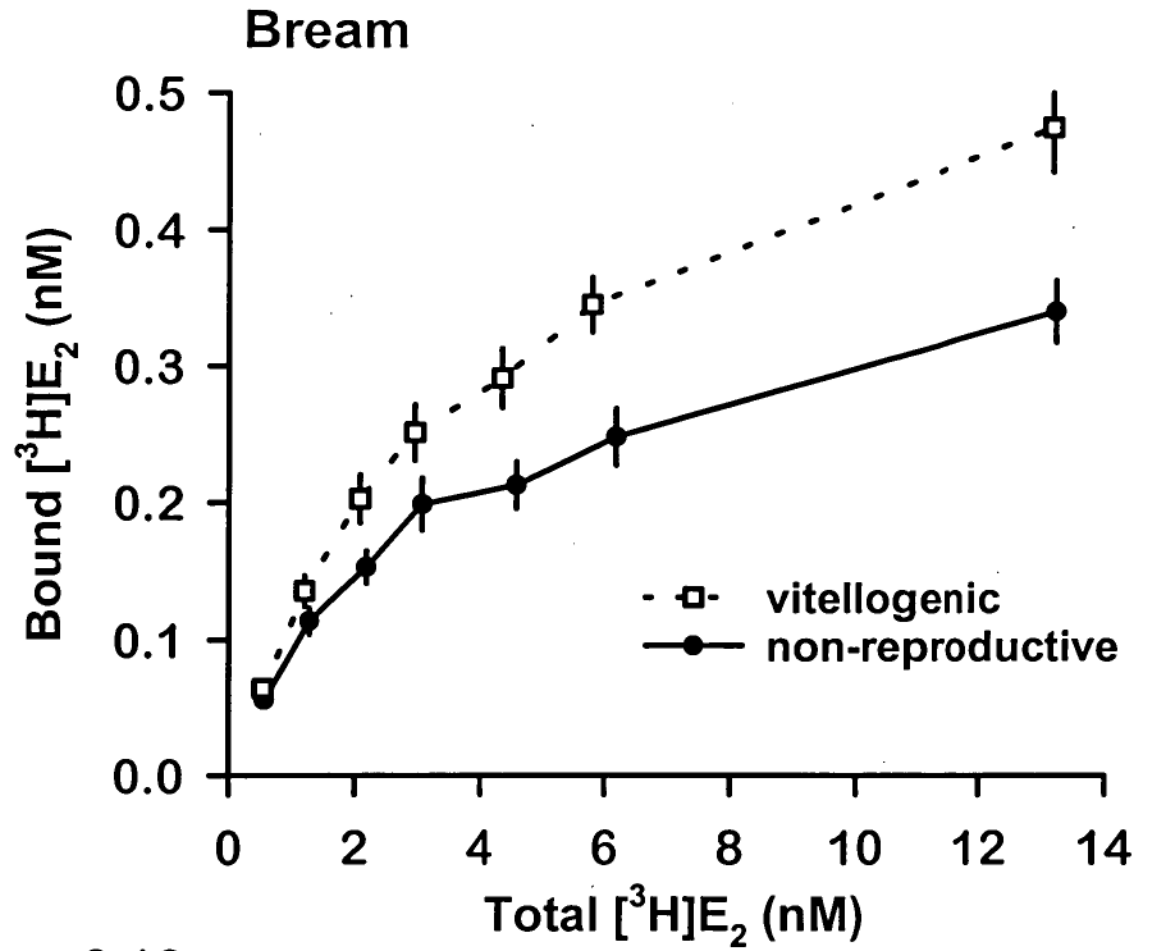
bream also differed with reproductive stage. Vitellogenic fish had greater capacity (60 nM) than non-reproductive fish (39 nM) (Table 5.2, Fig. 5.2). In contrast to the results from trout and bream, no differences in B_{max} were found between vitellogenic and non-reproductive flounder plasma (B_{max} ~ 400 nM; Table 5.2, Fig. 5.3). There were no significant differences in k_D with reproductive stage in any species (Table 5.3), although k_D appeared slightly higher in vitellogenic trout than in non-reproductive trout. Similarly, the relative affinity of binding of steroids compared to E_2 did not change with reproductive stage in trout (Table 5.4), bream (Table 5.5) or flounder (Table 5.6).

The k_D for E_2 binding in male trout plasma was significantly different between fish treated with E_2 (k_D = 1.34 nM) and saline (k_D = 0.43 nM; Table 5.7, Fig. 5.4). No changes in B_{max} were evident (B_{max} = 47 - 60 nM in all groups). Measurement of the absorbance after partial purification of plasma by gel filtration confirmed the presence of a protein (a putative vitellogenin) with a high molecular weight (> 460 kDa) that was not present in control fish (Fig. 5.5). To identify the fraction containing SBP, the total binding of each fraction to ^{3}H-E_2 was measured. E_2 binding in the control fish consisted of 2 peaks - a large peak centred around fraction 24, and a smaller peak around fractions 16 - 18 containing less than 10 % of the total binding (Fig. 5.5). A low level of binding to E_2 likely to be non-specific binding was also present in each fraction (Fig. 5.5). Total E_2 binding in E_2-injected fish exhibited a broader peak (Fig. 5.5). There was no binding of E_2 above background levels to fractions containing vitellogenin (Fig. 5.5). After partial purification by gel filtration, the SBP-containing fractions comprised 2 binding sites in both groups (Fig. 5.6). There were no significant differences between the binding characteristics of either the 1st or 2nd binding site between E_2-treated and saline-treated fish (Table 5.8). The molecular weight of trout SBP was estimated to be around 65 kDa (Fig 5.7).

5.4 Discussion

Differences were found between SBP characteristics of non-reproductive and reproductive trout and bream in the present study. B_{max} was higher in vitellogenic than non-reproductive fish in both trout and bream. Changes in B_{max} but not k_D with reproductive stage, as found in rainbow trout and black bream in the present study, have not previously been reported

Figure 5.2: Saturation curve (upper) and Scatchard (lower) plots for SBP in plasma of vitellogenic and non-reproductive bream. Points are mean ± se, n = 5 fish.


Bream
Bound [3H]E2 (nM)
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
Total [3H]E2 (nM)
vitellogenic
non-reproductive

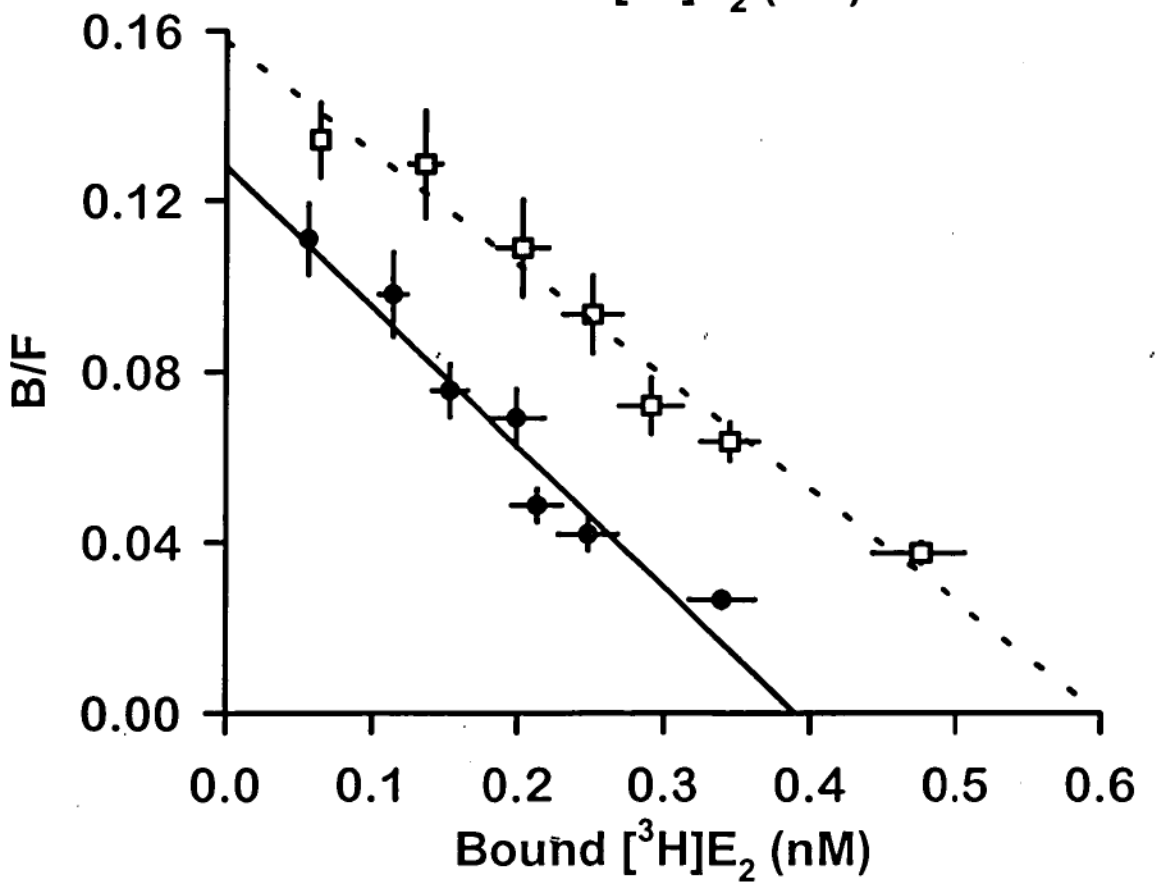
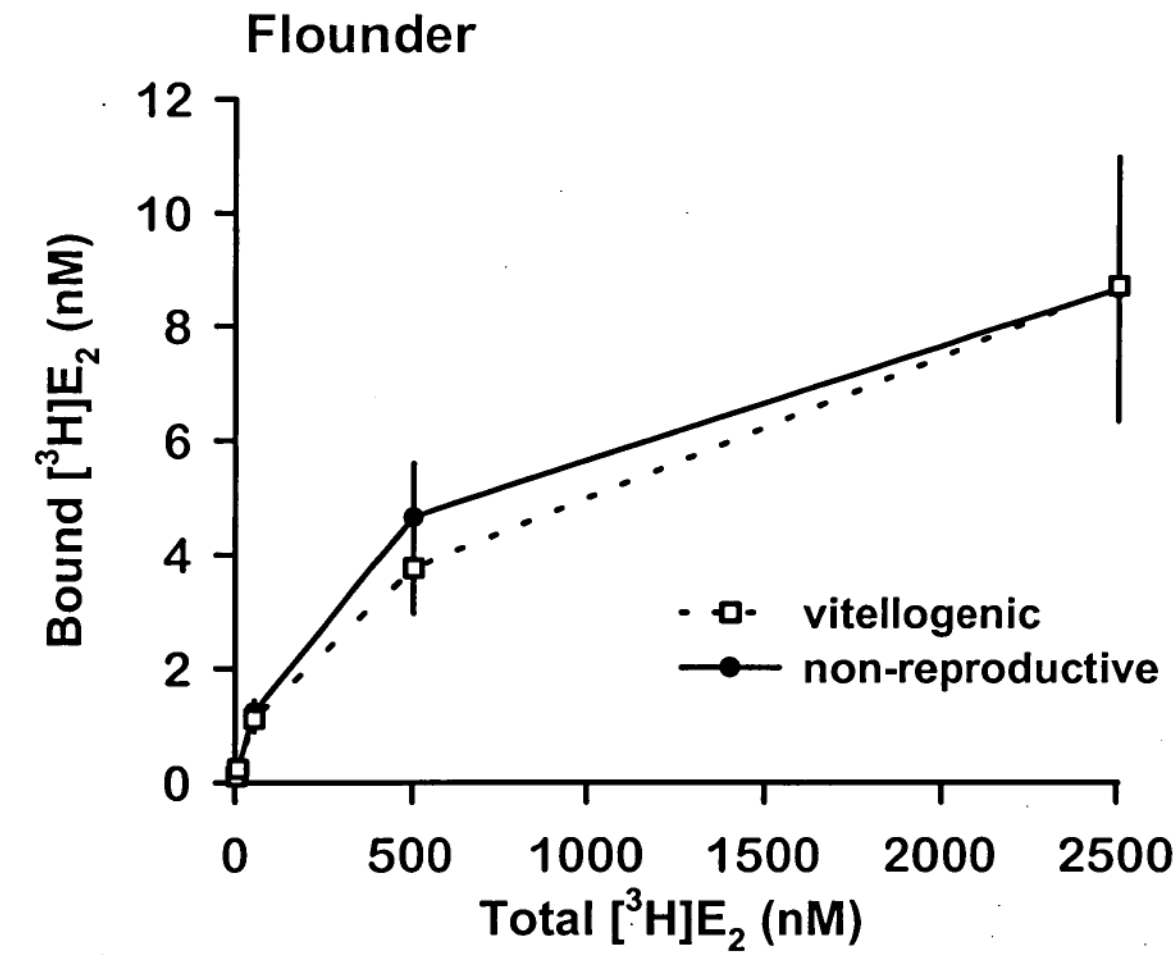

B/F
0.16
0.12
0.08
0.04
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Bound [3H]E2 (nM)

Figure 5.3: Saturation curve (upper) and Scatchard (lower) plots for SBP in plasma of vitellogenic and non-reproductive flounder. Points are mean ± se, n = 5 fish.


Flounder
Bound [3H]E2 (nM)
Total [3H]E2 (nM)
vitellogenic
non-reproductive
0
2
4
6
8
10
12
0
500
1000
1500
2000
2500

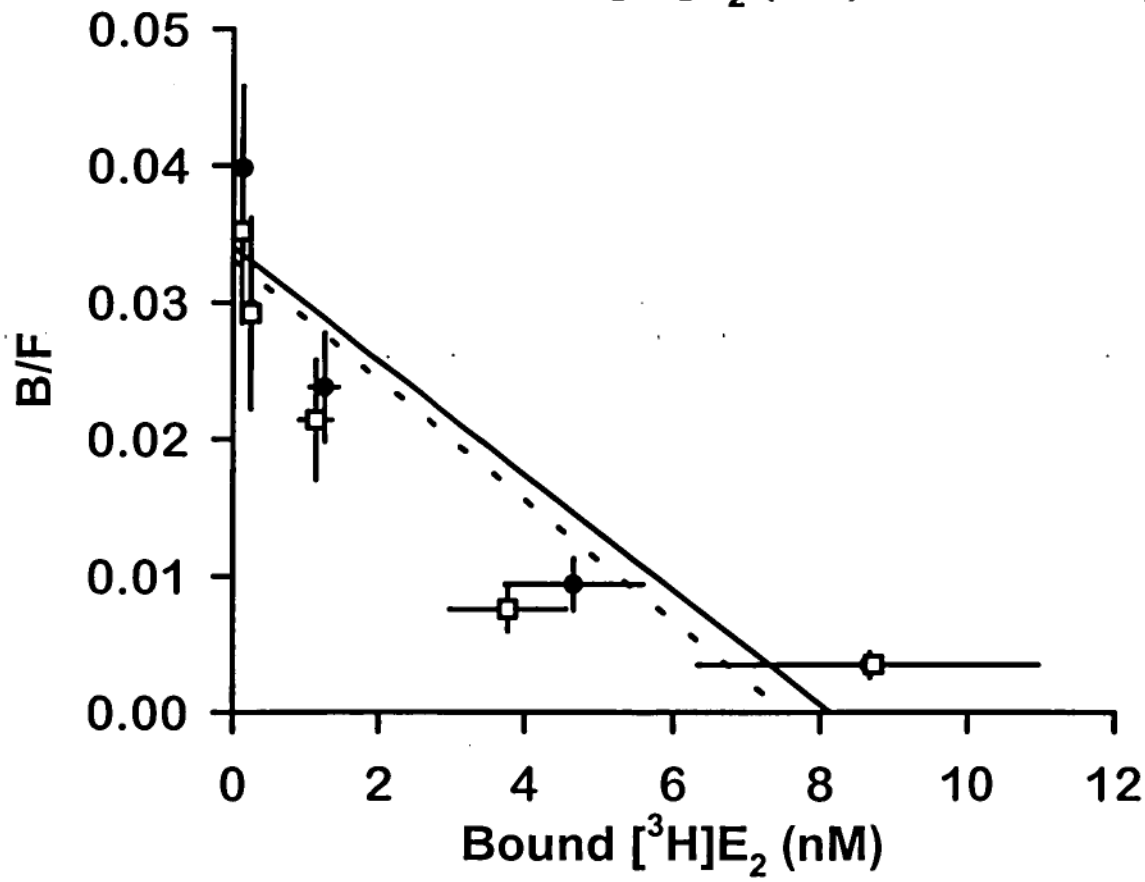

B/F
Bound [3H]E2 (nM)
0.00
0.01
0.02
0.03
0.04
0.05
0
2
4
6
8
10
12

Table 5.3: Mean binding affinity for non-reproductive and vitellogenic trout, bream and flounder, with upper and lower 95 % confidence limits calculated from estimates produced by LIGAND. Significant differences (non-overlapping confidence limits) between non-reproductive and vitellogenic fish are shown by *.

	Non-reproductive		Vitellogenic	
Species	k_D (nM)	(95 % CL)	k_D (nM)	(95 % CL)
trout	0.39	(0.22, 0.72)	0.65	(0.54, 0.79)
bream	3.05	(2.26, 4.10)	3.81	(2.86, 5.07)
flounder	239	(147, 390)	221	(128, 381)

Table 5.4: Binding characteristics of binding of E_2, T, cortisol and 17,20βP to SBP from non-reproductive and vitellogenic trout, and affinity of binding relative to E_2.

Steroid	k_D (nM)	k_D (95 % CL)	Maximum inhibition of binding (mean ± se)	Affinity relative to E_2 (%)	n
	Rainbow trout - non-reproductive fish				
E_2	0.58	(0.25, 1.35)	100	100	5
T	1.78	(0.83, 3.85)	>100	32.6	2
cortisol	3089	(1802, 5294)	67.3 ± 1.69	<0.1	4
17,20βP	_		31.5 ± 2.76	<0.1	5
	Rainbow trout - vitellogenic fish				
E_2	1.48	(0.90, 2.44)	100	100	5
T	4.38	(3.17, 6.06)	99.8 ± 1.09	33.8	5
cortisol	4703	(3590, 6160)	68.7 ± 0.92	<0.1	5
17,20βP	_		31.5 ± 1.77	<0.1	5

Table 5.5: Binding characteristics of binding of E_2, T, cortisol and 17,20βP to SBP from non-reproductive and vitellogenic bream, and affinity of binding relative to E_2.

Steroid	k_D (nM)	k_D (95 % CL)	Maximum inhibition of binding (mean ± se)	Affinity relative to E_2 (%)	n
	Black bream - non-reproductive fish				
E_2	3.90	(1.93, 7.89)	100	100	5
T	31.1	(19.6, 49.4)	>100	12.5	5
cortisol	1405	(930, 2123)	96.3 ± 0.66	0.3	5
17,20βP	1847	(1518, 2248)	92.2 ± 0.72	0.2	5
	Black bream - vitellogenic fish				
E_2	3.90	(1.17, 12.98)	100	100	5
T	32.8	(13.4, 80.5)	>100	11.9	5
cortisol	1271	(701, 2302)	93.9 ± 0.91	0.3	5
17,20βP	1815	(963, 3420)	89.7 ± 1.00	0.2	5

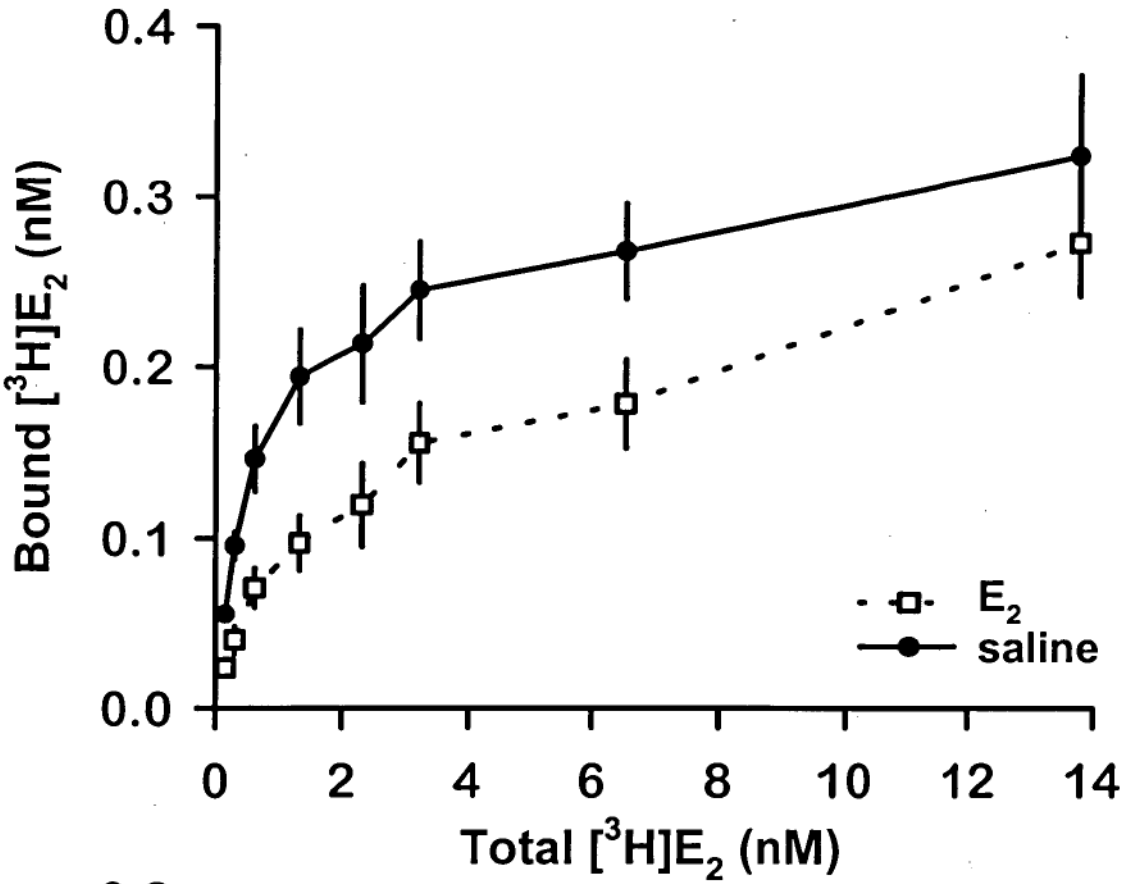
Table 5.6: Binding characteristics of binding of E_2, T, cortisol and 17,20βP to SBP from non-reproductive and vitellogenic flounder, and affinity of binding relative to E_2. (Values for E_2 are also presented in Tables 5.2 and 5.3.)

Steroid	k_D (nM)	k_D (95 % CL)	Maximum inhibition of binding (mean ± se)	Affinity relative to E_2 (%)	n
	Greenback flounder - non-reproductive fish				
T	95.2	(26.9, 336.4)	>100	418	5
E_2	(239)		100	100	(5)
cortisol	5840	(1848, 18451)	92.0 ± 2.99	6.8	5
17,20βP	1709	(709, 4119)	>100	23.3	5
	Greenback flounder - vitellogenic fish				
T	49.1	(4.2, 569.5)	>100	450	5
E_2	(221)		100	100	(5)
cortisol	4915	(455, 53068)	94.2 ± 2.59	4.5	5
17,20βP	434	(35, 5467)	99.1 ± 5.12	50.9	5

Table 5.7: Binding characteristics of male trout injected with either saline (controls) or E_2. Significant differences (non-overlapping 95 % confidence limits calculated from estimates from LIGAND) in k_D between the E_2 injected fish at the end of the experiment and all other groups are shown by different superscripts. There were no differences in B_{max}.

	Start of experiment			End of experiment		
	k_D (nM)	(95 % CL)	n	k_D (nM)	(95 % CL)	n
Control fish	0.61 ^{ab}	(0.27, 1.36)	5	0.43 ^{a}	(0.28, 0.68)	3
E_2-injected fish	0.67 ^{ab}	(0.40, 1.14)	5	1.34 ^{b}	(0.85, 2.12)	5
	B_{max} (nM)	(95 % CL)	n	B_{max} (nM)	(95 % CL)	n
Control fish	50	(36, 71)	5	60	(51, 71)	3
E_2-injected fish	58	(46, 72)	5	47	(37, 60)	5

Figure 5.4: Saturation curve (upper) and Scatchard (lower) plots for SBP in plasma of male trout treated with E_2 or saline for 3 weeks. Points are mean ± se, n = 5 fish.


0.4
0.3
0.2
0.1
0.0
Bound [^{3}H]E$_2$ (nM)
E$_2$
saline
0
2
4
6
8
10
12
14
Total [^{3}H]E$_2$ (nM)

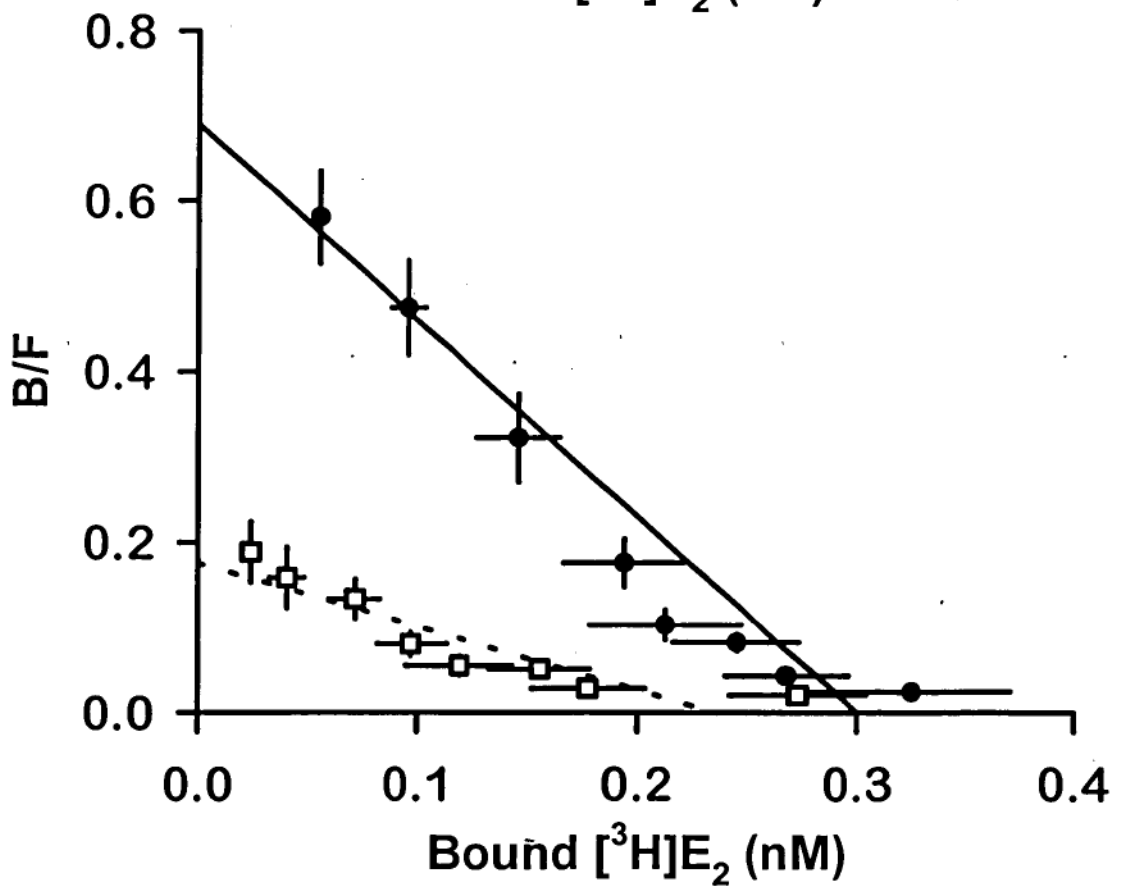

0.8
0.6
0.4
0.2
0.0
B/F
0.0
0.1
0.2
0.3
0.4
Bound [^{3}H]E$_2$ (nM)

Figure 5.5: Absorbance (lines) and total E_2 binding (bars) of fractions after partial purification of plasma from saline injected controls and E_2-treated male trout by gel filtration.

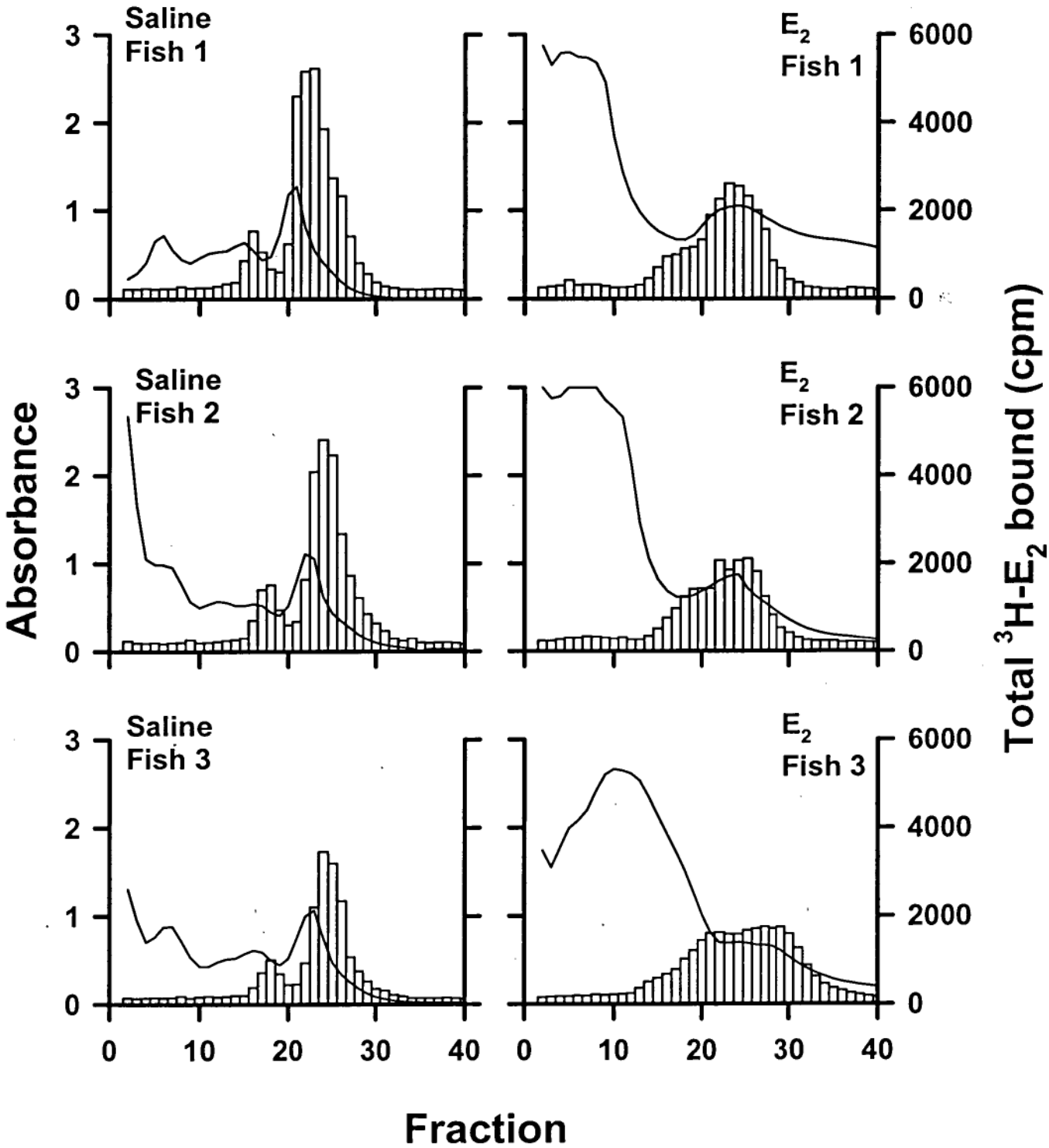
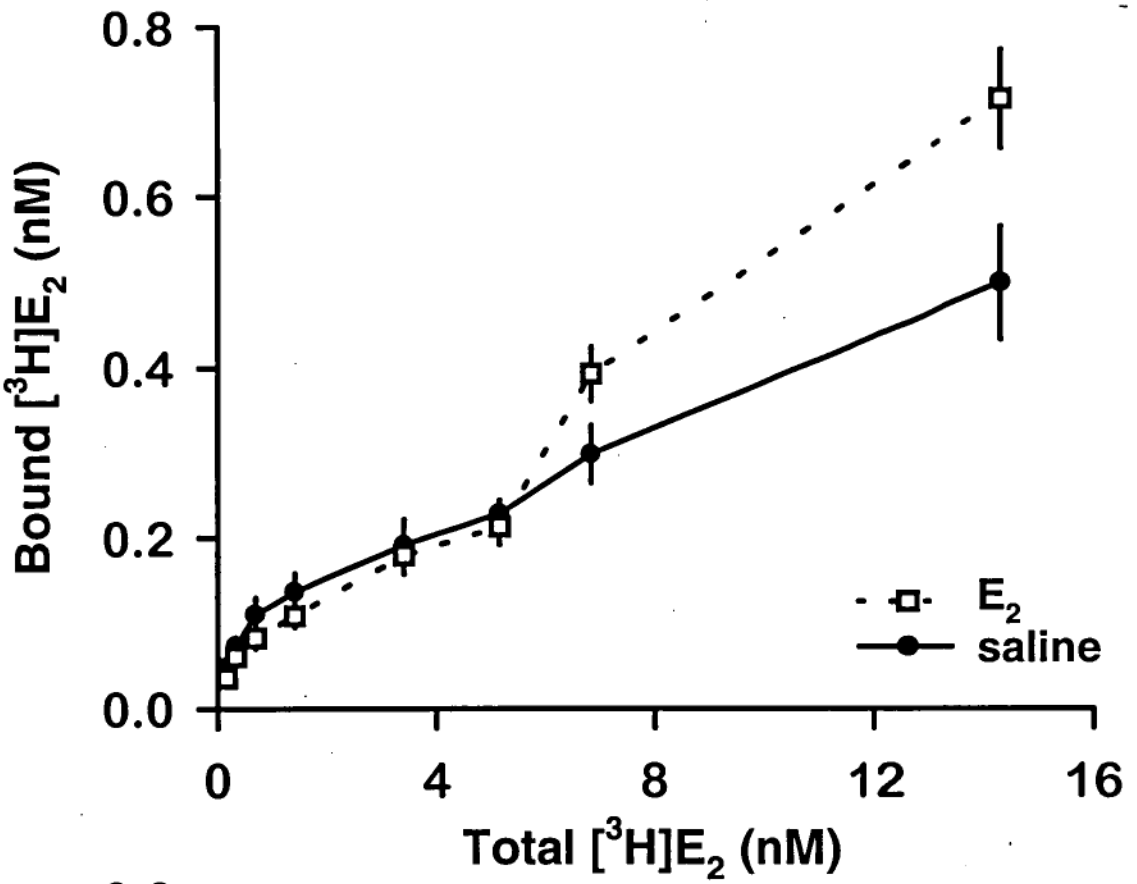

Saline
Fish 1
E_2
Fish 1
Saline
Fish 2
E_2
Fish 2
Saline
Fish 3
E_2
Fish 3
Absorbance
Total ^3H-E_2 bound (cpm)
Fraction
0
1
2
3
0
2000
4000
6000
0
10
20
30
40

Figure 5.6: Saturation curve (upper) and Scatchard (lower) plots for SBP partially purified by gel filtration from plasma of male trout treated with E_2 or saline for 3 weeks. Points are mean ± se, n = 3 fish.


0.8
0.6
0.4
0.2
0.0
Bound [^{3}H]E_2 (nM)
E_2
saline
0
4
8
12
16
Total [^{3}H]E_2 (nM)

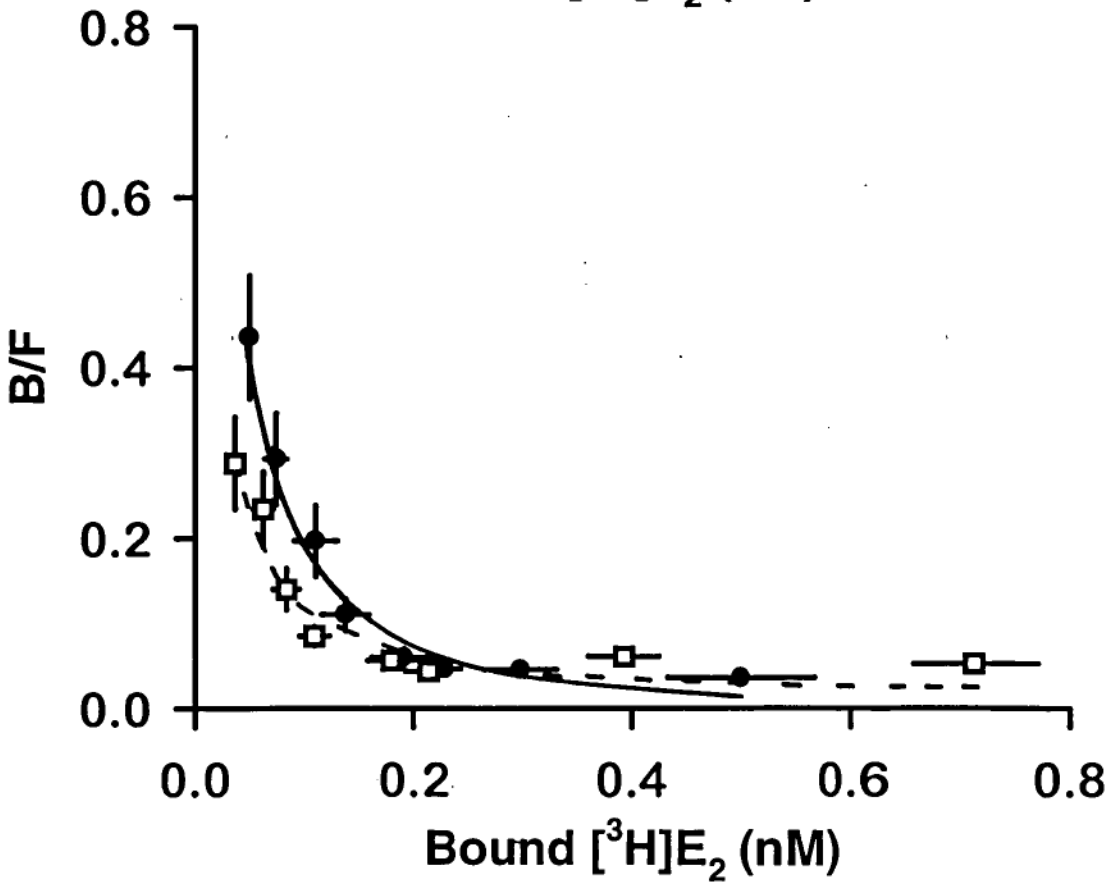
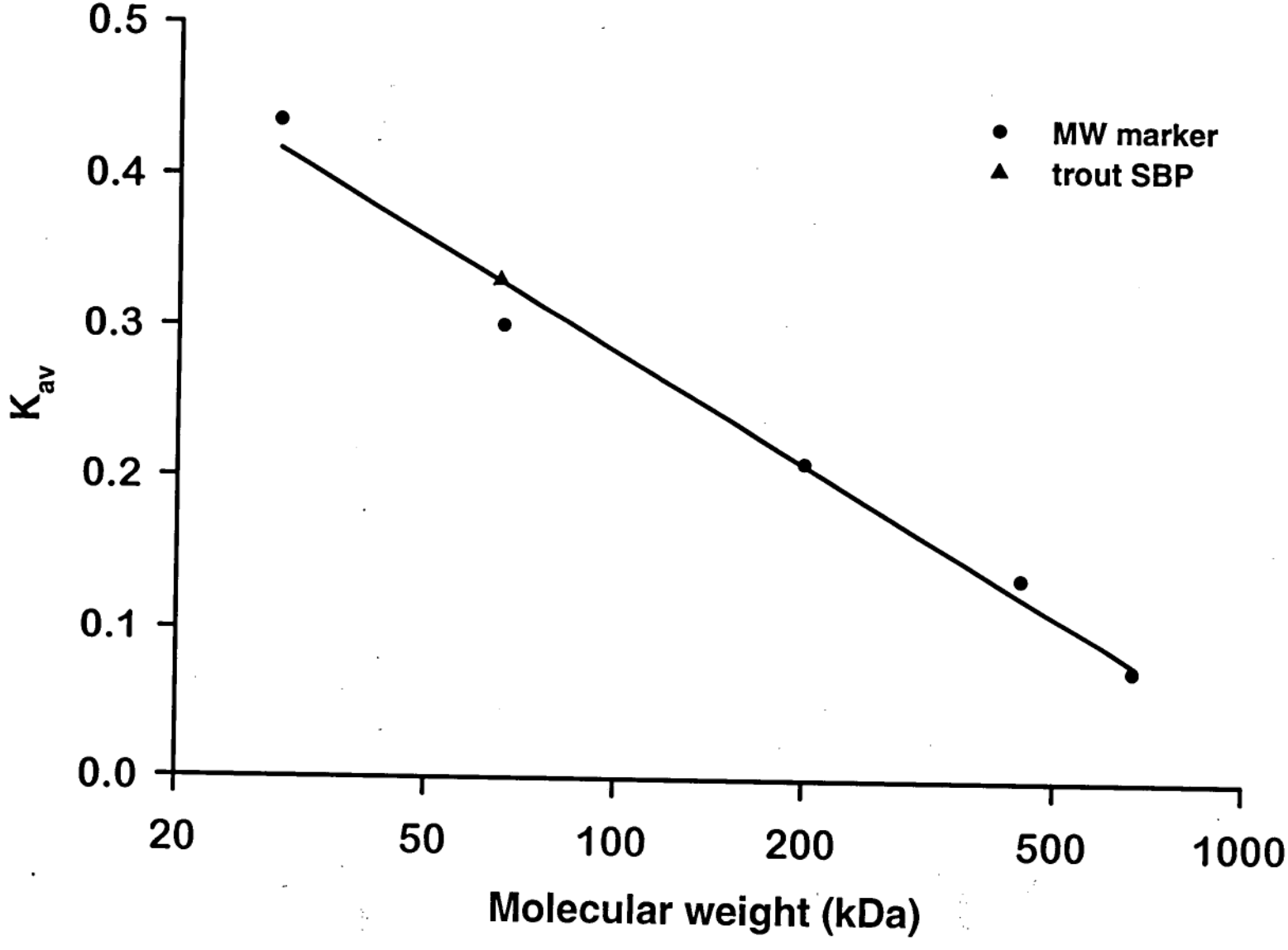

0.8
0.6
0.4
0.2
0.0
B/F
0.0
0.2
0.4
0.6
0.8
Bound [^{3}H]E_2 (nM)

Table 5.8: Binding characteristics of plasma from male trout injected with either saline or E_2, after partial purification of SBP by gel filtration. There were no significant differences.

	k_D site 1 (95 % CL) (nM)	k_D site 2 (95 % CL) (nM)	n
Control fish	0.17 (0.03, 0.88)	218 ($1.1x10^{-7}$, $4.2x10^{11}$)	3
E_2-injected fish	0.11 (0.01, 1.28)	187 ($7.0x10^{-4}$, $5.2x10^{7}$)	3
	B_{max} site 1 (95 % CL) (nM)	B_{max} site 1 (95 % CL) (nM)	
Control fish	23 (10, 50)	1232 ($2.8x10^{-6}$, $5.4x10^{11}$)	3
E_2-injected fish	12 (4, 32)	1688 ($1.7x10^{-2}$, $1.6x10^{8}$)	3

Figure 5.7: Calibration curve for determination of trout SBP molecular weight by gel filtration. The markers used were carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), β-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).


0.5
0.4
0.3
0.2
0.1
0.0
K_{av}
MW marker
trout SBP
20
50
100
200
500
1000
Molecular weight (kDa)

for any female teleosts. The magnitude of the increase in B_{max} in vitellogenic fish found in the present study (61 % for trout and 54 % for bream) is similar to the increase of about 50 % in B_{max} found in spotted seatrout undergoing final oocyte maturation compared to regressed fish (Laidley and Thomas, 1997). An increase in k_D, however, was also observed in spotted seatrout concomitant with the change in B_{max} (Laidley and Thomas, 1997). A higher k_D was also found in female goldfish with high GSI compared to females with low GSI (Van Der Kraak and Biddiscombe, 1999). Results similar to the present study have been found in the female green frog (Paolucci and Di Fiore, 1994) where the highest concentration of SBP was present concurrently with the highest plasma E_2 levels. This is opposite to what is generally seen in male vertebrates. In male brown trout, SBP decreases when T concentrations peak (Pottinger, 1988). In other vertebrates, there is also a trend in males for reduced circulating levels of SBP when plasma T is highest (Saboureau *et al.*, 1982; Paolucci *et al.*, 1992), or no change in SBP and plasma T levels in excess of B_{max} at some stages (Moore *et al.*, 1983; Ho *et al.*, 1987).

In contrast to trout and bream, no differences in B_{max} or k_D were found between vitellogenic and non-reproductive flounder. Similar results have been reported for goldfish by Pasmanik and Callard (1986), where no seasonal or sex differences in k_D or B_{max} were found. No correlation was found between plasma T or E_2 levels and B_{max} of the female common carp (Chang and Chen, 1991), however, the B_{max} in carp did vary by up to 100 %, and the reproductive stage of the fish at each sampling time was not assessed. The only other flatfish for which any mention of SBP is made is the winter flounder *Pseudopleuronectes americanus*, where a single measurement for SBP activity in prespawning fish did not differ from that for vitellogenic fish (Nagler and Idler, 1992).

The lack of change of any binding characteristics of flounder SBP with reproductive stage suggests that the increased plasma levels of E_2 and T are able to induce the appropriate responses without any requirement for extra protection either for or from the steroids. The low affinity of the flounder SBP relative to that of trout and bream (Chapter 3, this volume) also suggests a lesser importance of SBP in flounder reproduction. In contrast, the increased binding capacity of bream SBP in vitellogenic fish supports the idea of a protective role for SBP, with higher plasma steroid levels being maintained by more SBP. The binding capacity of SBP is almost always sufficient to bind all circulating steroid in

most species investigated (Callard and Callard, 1987), and this appears to be the case for bream also (Chapter 3, this volume). Therefore an increased binding capacity to simply protect more steroid seems unnecessary. If the main function of SBP is as a reservoir for steroids, however, then buffer systems that are far from saturation in usual conditions are more effective (Mendel, 1989). Cell surface receptors for SBP also exist in some mammals (Hryb *et al.*, 1990). The unliganded SBP binds to the receptor, before accepting ligand (E_2 or T), and activating a cellular response (Rosner *et al.*, 1992). If receptors of this type are present in teleosts, it is possible that a high ratio of SBP to steroid is required to ensure sufficient concentrations of unliganded SBP are available for binding to the cell surface receptors.

In contrast, the B_{max} of SBP in vitellogenic trout is insufficient to bind all circulating E_2 and T at the peak levels that have been measured for these steroids (B_{max} = 124 nM, the present study; E_2 = 30 ng.ml^{-1} (110 nM) and T = 140 ng.ml^{-1} (485 nM), Pankhurst and Thomas, 1998). Although peak plasma E_2 and T levels in trout reach 30 and 140 ng.ml^{-1} respectively (Pankhurst and Thomas, 1998)), the levels of E_2 and T measured in fish used in the present study were about 19 and 10 ng.ml^{-1} respectively (Table 5.1), suggesting mid rather than late vitellogenesis. These concentrations are less than the B_{max} measured in these fish, and consistent with the general trend of steroid levels lower than the B_{max} (Callard and Callard, 1987). It is possible that a further increase in SBP levels may occur, such that the binding capacity is still sufficient to protect the peak concentrations of E_2 and T that have been measured in this stock of fish.

In an attempt to reproduce the conditions in female vitellogenic trout, male trout in the present study were injected with E_2. The values determined for k_D and B_{max} of the male fish prior to the experiment (k_D = 0.61 - 0.67 nM, B_{max} = 50 - 58 nM) were similar to those found in non-reproductive female trout (k_D = 0.39 nM, B_{max} = 77 nM). This is consistent with the trend in other animals for similar k_D in both sexes, and similar or slightly higher B_{max} in females (Martin, 1975; Martin and Ozon, 1975; Pottinger, 1986; Ho *et al.*, 1987: Laidley and Thomas, 1997). The difference in B_{max} between non-reproductive and vitellogenic female fish was not reproduced by treatment of male trout with E_2. This is in contrast to the results reported by Foucher *et al.* (1991) who found a significant increase in SBP levels in male rainbow trout 14 days after injection with E_2. A second injection 14

days after the first also resulted in a further increase in SBP levels 7 days after the second injection (Foucher *et al.*, 1991). The reason for the differences between the 2 studies in unclear. The fish in both studies were nonspermiated adult males and the doses of E_2 were of a similar order of magnitude (5 $mg.kg^{-1}$, the present study; 0.5 $mg.kg^{-1}$ (1st injection) and 1 $mg.kg^{-1}$ (2nd injection), Foucher *et al.*, 1991).

Although B_{max} was not different between E_2-treated and control rainbow trout in the present study, k_D differed significantly between the 2 groups. The decrease in binding affinity apparent in the E_2-injected fish compared to the saline-injected fish was similar to the non-significant decrease in affinity (increase in k_D) found in the vitellogenic compared to non-reproductive female trout. It has previously been suggested that decreased binding affinity found in reproductive compared to regressed female fish may be an artefact of the increased plasma levels of vitellogenin (Laidley and Thomas, 1997). To determine whether the difference in k_D found in the male trout resulted from interference of plasma vitellogenin, the SBP was partially purified to remove vitellogenin. From the measurement of the absorbance and total binding in each fraction after gel filtration, 2 points were immediately apparent. Firstly, ^3H- E_2 does not appear to bind to vitellogenin. Therefore, even though the change in B_{max} was not replicated in the experimental male trout, the increase in B_{max} in vitellogenic female trout is unlikely to be a result of binding to vitellogenin. Secondly, the curve of total ^3H- E_2 binding in each fraction differed between saline-treated and E_2-treated fish. In the saline-treated fish, there was a peak of E_2 binding around fractions 22 to 25, and a smaller peak around fractions 16 to 18. The fact that the 2 peaks are relatively distinct suggests that the smaller peak may consist of an albumin-like protein responsible for the low affinity, high capacity binding to E_2, as shown by albumin in mammals (Westphal, 1986). Although albumin has not been found in fish, proteins with some of the characteristics of albumin are present (Davidson *et al.*, 1989; Maillou and Nimmo, 1993a, b). Binding of steroid hormones to these albumin-like proteins, however, has not been investigated. The fractions showing binding to E_2 in the E_2-treated fish do not show 2 distinct peaks. A possible explanation for this is interference of the very high levels of vitellogenin with the later elution of smaller molecular weight proteins including SBP off the gel filtration column. This could result in less separation between peaks of lower molecular weight proteins.

After partial purification by gel filtration, the Scatchard plots of data from both the saline and E_2-treated fish (Fig. 5.6) were demonstrably different from plots from the assays of whole plasma (Fig. 5.4). There were 2 main differences. Firstly, and most importantly, the difference in k_D between the experimental groups was no longer present. This suggests that the difference in k_D found in this experiment probably resulted from interference of high concentrations of high molecular weight proteins (primarily vitellogenin), and not from a change in the k_D of SBP. The slight, but not significant difference in k_D between vitellogenic and non-reproductive female trout is, therefore, also likely to be related to high vitellogenin concentrations. It is also possible, however, that the difference in k_D between the 2 groups before partial purification of the plasma, resulted from other factors present in the plasma that did not elute in the same fractions as SBP. Polyunsaturated fatty acids have been shown to competitively inhibit binding of E_2 to SBP in goldfish, resulting in a decrease in the affinity of E_2 for SBP (Van Der Kraak and Biddiscombe, 1999). Plasma levels of fatty acids vary throughout reproductive development (Ballantyne *et al.* 1996), and the difference in k_D found in the present study may be related to changing fatty acid profiles, and not changing vitellogenin concentrations.

Further indirect evidence that any difference measured in k_D is not due to a change in SBP binding affinity is provided by the assays of relative affinities of other steroids (T, 17,20βP and cortisol) for SBP. There were no differences in the relative affinities of these steroids for SBP in vitellogenic compared to non-reproductive fish. However, changes in the binding affinity of other steroid ligands would need to be large for a difference to be significant, as there is more inherent variation present in competitive binding assays with low affinity ligands. Secondly, binding to E_2 after gel filtration appears to consist of 2 binding sites rather than the single site apparent from saturation assays of diluted whole plasma. The first binding site is a high affinity (k_D = 0.11 - 0.17 nM), low capacity (B_{max} = 12 - 23 nM) binding site most likely to be SBP. The second binding site is much lower in affinity (k_D = 187 - 218 nM) and considerably higher in capacity (B_{max} = 1.2 - 1.7 μM). The estimates of k_D and B_{max} for the second binding site have been obtained by extrapolation of the binding curve above E_2 levels used in the assays, resulting in the sizeable confidence limits reported (Table 5.8). Accepting this proviso, it is also clear that the straight line fit of a single binding site is inadequate for this data. The possible identity

of this second binding site is uncertain. It appears that trout do have protein in the plasma that shares some characteristics (low affinity, high capacity) of albumin binding in mammals. However, whether this protein is one of the albumin-like proteins found in trout plasma by Maillou and Nimmo (1993a) will remain uncertain until binding of steroids to these albumin-like proteins is investigated. If both the albumin-like proteins in trout plasma bind steroids, this could explain the small peak of ^{3}H-E_2 binding around fractions 16 to 18 (Fig. 5.5), and also the second binding site found after assay of partially purified plasma where fractions containing the smaller peak were excluded.

The molecular weight of SBP has only been reported for 3 teleost species to date, and ranges from 64 kDa for the eel *Anguilla japonica* (Chang *et al.*, 1994) to 194 kDa for the carp (Chang and Lee, 1992). The molecular weight of rainbow trout SBP from the present study was estimated to be around 65 kDa by gel filtration after partial purification. Molecular weights of the same molecule can differ when estimated using different methods. The molecular weight of carp and eel SBPs were both determined by HPLC after purification of the molecule, suggesting that substantial differences in the size of the SBP of these 2 species are real. Estimates of spotted seatrout SBP differed when measured by gel filtration (around 150 kDa) and native PAGE (approximately 135 kDa) in the same study (Laidley and Thomas, 1994). Gel filtration after partial purification is not an optimal method for determining molecular weight, but does provide an initial estimate for rough comparative purposes. An estimate of 65 kDa suggests rainbow trout SBP may be more similar to eel than carp SBP.

In conclusion, B_{max} is higher in vitellogenic than non-reproductive female rainbow trout and black bream. This difference in trout does not appear to result directly from elevated plasma E_2 and vitellogenin levels experienced by the fish at this time. Interference of high concentrations of vitellogenin, however, may generate small changes in k_D. In greenback flounder, the binding characteristics of SBP remain unchanged at these reproductive stages, suggesting a lesser role for SBP in maintaining elevated plasma levels of steroids in this species.

5.5 References

Ballantyne, J.S., Mercure, F., Gerrits, M.F., Van Der Kraak, G., McKinley, S., Martens, D.W., Hinch, S.G., and Diewert, R.E. (1996). Plasma nonesterified fatty acid profiles in male and female sockeye salmon, *Oncorhynchus nerka*, during the spawning migration. *Can. J. Fish. Aquat. Sci.* **53**, 1418-1426.

Barnett, C.W. (1998). Reproductive biology and endocrinology of the greenback flounder *Rhombosolea tapirina* (Günther, 1862). PhD thesis, University of Tasmania, Launceston.

Barnett, C.W., and Pankhurst, N.W. (1999). Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther 1862). *Mar. Freshwater Res.* **50**, 35-42.

Baulieu, E.E. (1986). Steroid hormone binding proteins and their intra- and extra-cellular congeners. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 1-11. John Libbey Eurotext, London.

Bordin, S., and Petra, P.H. (1980). Imunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey *Macaca nemestrina*. *Proc. Natl. Acad. Sci. USA* **77**, 5678-5682.

Callard, I.P., and Callard, G.V. (1987). Sex steroid receptors and non-receptor binding proteins. *In* "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D.O. Norris, and R.E. Jones, Eds.), pp. 355-384. Plenum Press, New York.

Carragher, J.F., and Pankhurst, N.W. (1993). Plasma levels of sex steroids during sexual maturation of snapper, *Pagrus auratus* (Sparidae), caught from the wild. *Aquaculture* **109**, 375-388.

Chang, C.F., and Chen, M.R. (1990). Fluctuations in sex steroids and sex steroid-binding protein during the development and annual cycle of the male common carp, Cyprinus carpio. *Comp. Biochem. Physiol.* **97A**, 565-568.

Chang, C.F., and Chen, M.R. (1991). Lack of correlation found between plasma sex steroids and sex steroid-binding capacity during the annual cycle of the female common carp. *Bull. Inst. Zool., Academia Sinica* **30**, 81-86.

Chang, C.F., and Lee, Y.H. (1992). Purification of the sex steroid-binding protein from common carp (*Cyprinus carpio*) plasma. *Comp. Biochem. Physiol.* **101B**, 587-590.

Chang, C.F., Lee, Y.H., Yoshida, T., and Sun, L.T. (1994). Characterization of the plasma sex steroid-binding protein in eel (*Anguilla japonica*). *Comp. Biochem. Physiol.* **108B**, 189-197.

Davidson, W.S., Bartlett, S.E., Birt, T.P., Birt, V.L., and Green, J.M. (1989). Identification and purification of serum albumin from rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **93B**, 5-9.

Foucher, J.L., Niu, P.D., Mourot, B., Vaillant, C., and Le Gac, F. (1991). *In vivo* and *in vitro* studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Molec. Biol.* **39**, 975-986.

Foucher, J.L., Le Bail, P.-Y., and Le Gac, F. (1992). Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **85**, 101-110.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Ho, S.M., Lance, V., and Megaloudis, M. (1987). Plasma sex-steroid binding protein in a seasonally breeding reptile, *Alligator mississippiensis*. *Gen. Comp. Endocrinol.* **65**, 121-132.

Hobby, A.C., and Pankhurst, N.W. (1997). Comparative aspects of steroid binding protein (SBP) in four fishes: a salmonid, a pleuronectid and two sparids. *In* "Advances in Comparative Endocrinology" (S. Kawashima, and S. Kikuyama, Eds.), pp. 1369-1373. Monduzzi Editore, Bologna.

Hobby, A.C., Pankhurst, N.W., and Haddy, J.A. (2000). The effects of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology* **125A**, 85-94.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1986). Specific binding of human corticosteroid-binding globulin to cell membranes. *Proc. Natl. Acad. Sci. USA* **83**, 3253-3256.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048-6054.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Laidley, C.W., and Thomas, P. (1997). Changes in plasma sex steroid-binding protein levels associated with ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **56**, 931-937.

Maillou, J., and Nimmo, I.A. (1993a). Albumin-like proteins in the serum of rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **104B**, 387-393.

Maillou, J., and Nimmo, I.A. (1993b). Identification and some properties of an albumin-like protein in the serum of pre-spawning Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol.* **104B**, 401-405.

Martin, B. (1975). Steroid-protein interactions in nonmammalian vertebrates. I. Elasmobranch steroids-binding protein (E.SBP) in dogfish serum (*Scyliorhinus canicula*). *Gen. Comp. Endocrinol.* **25**, 42-51.

Martin, B., and Ozon, R. (1975). Steroid-protein interactions in nonmammalian vertebrates. II. Steroids binding proteins in the serum of amphibians; a physiological approach. *Biol. Reprod.* **13**, 371-380.

McPherson, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacological Methods* **14**, 213-228.

Mendel, C.M. (1989). The free hormone hypothesis: a physiologically based mathematical model. *Endocr. Rev.* **10**, 232-274.

Moore, F.L., Spielvogel, S.P., Zoeller, R.T., and Wingfield, J. (1983). Testosterone-binding in a seasonally breeding amphibian. *Gen. Comp. Endocrinol.* **49**, 15-21.

Munson, P.J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.

Nagler, J.J., and Idler, D.R. (1992). *In vitro* ovarian estradiol-17β and testosterone responses to pituitary extract and corresponding serum levels during the prespawning to vitellogenic phases of the reproductive cycle in winter flounder (*Psuedopleuronectes americanus*). *Comp. Biochem. Physiol.* **101A**, 69-75.

Norris, D.O. (1997). "Vertebrate Endocrinology", p. 634. Academic Press, San Diego.

Pankhurst, N.W. (1998). Reproduction. *In* "Biology of Farmed Fish" (K.D. Black, and A.D. Pickering, Eds.), pp. 1-26. Academic Press, Sheffield.

Pankhurst, N.W., and Carragher, J.F. (1991). Seasonal endocrine cycles in marine teleosts. *In* "Reproductive Physiology of Fish 1991" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 131-135. FishSymp91, Sheffield.

Pankhurst, N.W., and Thomas, P.M. (1998). Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. *Aquaculture* **166**, 163-177.

Pankhurst, N.W., and Van Der Kraak, G. (1997). Effects of stress on reproduction and growth of fish. *In* "Fish Stress and Health in Aquaculture" (G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, Eds.), pp. 73-93. Cambridge University Press, Cambridge.

Paolucci, M., and Di Fiore, M.M. (1994). Sex steroid binding proteins in the plasma of the green frog, *Rana esculenta*: Changes during the reproductive cycle and dependence on pituitary gland and gonads. *Gen. Comp. Endocrinol.* **96**, 401-411.

Paolucci, M., De Fiore, M.M., Ciarcia, G., and Botte, V. (1992). Plasma sex steroid binding proteins (SSBP) in the male lizard, *Podarcis s. sicula*, during the reproductive cycle. *Gen. Comp. Endocrinol.* **87**, 232-239.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Molec. Biol.* **40**, 735-753.

Petra, P.H., Stanczyk, F.Z., Senear, D.F., Namkung, P.C., Novy, M.J., Ross, J.B.A., Turner, E., and Brown, J.A. (1983). Current status of the molecular structure and function of the plasma sex steroid-binding protein (SBP). *J. Steroid Biochem.* **19**, 699-706.

Porto, C.S., Musto, N.A., Bardin, C.W., and Gunsalus, G.L. (1992). Binding of an extracellular steroid-binding globulin to membranes and soluble receptors from human breast cancer cells (MCF-7 cells). *Endocrinology* **130**, 2931-2936.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1988). Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **70**, 334-344.

Rosner, W., Hryb, D.J., Khan, M.S., Nakhla, A.M., and Romas, N.A. (1992). Sex hormone-binding globulin: Binding to cell membranes and generation of a second messenger. *J. Androl.* **13**, 101-106.

Saboureau, M., Laurent, A.-M., and Boissin, J. (1982). Plasma testosterone binding protein capacity in relation to the annual testicular cycle in a hibernating mammal, the hedgehog (*Erinaceus europaeus* L.). *Gen. Comp. Endocrinol.* **47**, 59-63.

Scott, A.P., Bye, V.J., and Baynes, S.M. (1980). Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson). *J. Fish Biol.* **17**, 587-592.

Scott, A.P., and Canario, A.V.M. (1987). Status of oocyte maturation-inducing steroids in teleosts. *In* "Reproductive Physiology of Fish 1987" (D.R. Idler, L.W. Crim, and J.M. Walsh, Eds.), pp. 224-234. Memorial University of Newfoundland, St. Johns.

Stanczyk, F.Z., Namkung, P.C., Fritz, M.A., Novy, M.J., and Petra, P.H. (1986). The influence of sex steroid-binding protein on the metabolic clearance rate of testosterone. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 555-563. John Libbey Eurotext, London.

Thomas, P.M., Pankhurst, N.W., and Bremner, H.A. (1999). The effect of stress and exercise on post-mortem biochemistry of Atlantic salmon and rainbow trout. *J. Fish Biol.* **54**, 1177-1196.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

Ventling, A.R., and Pankhurst, N.W. (1995). Effects of gonadal steroids and human chorionic gonadotropin on final oocyte maturation *in vitro* in the New Zealand snapper *Pagrus auratus* (Sparidae). *Mar. Freshwater Res.* **46**, 467-473.

Westphal, U. (1986). "Steroid-Protein Interactions II", p. 592. Monographs on Endocrinology 27, Springer-Verlag, New York.

6. The effect of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*)

6. The effect of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*)

6.1 Introduction

The inhibitory effect of stress on reproductive processes in fish has been well established. Stress reduces circulating androgens and estrogens in many species and is also associated with increased ovarian atresia (Pankhurst and Van Der Kraak, 1997) probably by the process of apoptosis (Hsueh *et al.*, 1994). Reproduction may be impaired, or there may be complete shut down of reproduction for the current season in some species captured from the wild and maintained in captivity (Carragher and Pankhurst, 1991). Even in species where reproductive maturity is reached, there may still be significant effects on the progeny. For example, rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) subjected to repeated acute stress or confinement stress during maturation produce smaller and fewer eggs, which have a lower fertilisation rate and lower larval survival (Campbell *et al.*, 1992, 1994).

The mechanism by which stress acts to inhibit reproduction has not been determined. Several possible mechanisms exist, including a direct effect of cortisol at one or more sites in the endocrine cascade, or an effect from some other component of the secondary response to stress. Despite early studies suggesting cortisol was acting directly on ovarian tissue to reduce production of estradiol (E_2) and testosterone (T) in female salmonids (Carragher and Sumpter, 1990), inconsistent or negative results from subsequent studies on salmonids (Pankhurst, 1998; Pankhurst *et al.*, 1995a) and non-salmonids (Pankhurst *et al.*, 1995b) suggested stress was not acting via a direct effect of cortisol on ovarian steroidogenesis. At higher levels in the endocrine pathway, secretion of gonadotropin from the pituitary is reduced by cortisol treatment in female brown trout (Carragher *et al.*, 1989), but increases in gonadotropin secretion have also been shown (Pankhurst and Van Der Kraak, 1997). Despite the effect of stress on egg quality (Campbell *et al.*, 1992, 1994), this does not appear to result directly from the action of cortisol (Brooks *et al.*, 1995). Cortisol

may be having an indirect effect on reproduction by an as yet undetermined mechanism, or the inhibition of reproduction may be mediated by other aspects of the stress response (Sumpter *et al.*, 1994).

Alternatively, there may be an effect of stress on post-secretion events such as metabolism and clearance of steroid hormones from the plasma. Sex steroid binding protein (SBP) is a large molecule circulating in the plasma that protects estrogens and androgens (primarily E_2 and T) from metabolism, aids transport of these steroids to their target tissues and may also be involved in the passage of these steroids across the cell membrane (Hammond, 1990; Joseph, 1994). Any aspect of stress that changes the amount of SBP or its binding characteristics, has the potential to affect plasma steroid concentrations. It has been previously shown that cortisol implantation can affect SBP. Pottinger and Pickering (1990) showed a 33 % increase in the plasma binding capacity for E_2 two weeks after cortisol implantation in rainbow trout. However, there are no studies investigating the effect of stress on SBP over the 24 h period where plasma steroid levels of E_2 and T show a dramatic decline following capture and confinement in many species including rainbow trout (Pankhurst and Dedual, 1994), snapper (*Pagrus auratus*) (Carragher and Pankhurst, 1991) and red gurnard (*Chelidonichthys kumu*) (Clearwater and Pankhurst, 1997).

In the present study, we investigated the effect of confinement stress on SBP characteristics in two teleosts, a representative non-salmonid (black bream, *Acanthopagrus butcheri*) and a representative salmonid, rainbow trout. Black bream show a stress response that is typical of many non-salmonid teleosts. Cortisol levels increase from around 5 $ng.ml^{-1}$ within 5 min of capture to about 40 $ng.ml^{-1}$ within 1 h of confinement (Haddy and Pankhurst, 1999). The levels of E_2 and T drop significantly after 1 h, and cortisol remains above 20 $ng.ml^{-1}$ after 24 h while E_2 and T remain low. Rainbow trout show a larger elevation of cortisol in response to stress (35 - 900 $ng.ml^{-1}$, Barton and Iwama, 1991) but a less consistent or extreme suppression of gonadal steroids (Pankhurst and Van Der Kraak, 1997). The relative affinity of SBP for cortisol was also investigated to determine whether high plasma concentrations of cortisol associated with stress could compete with E_2 and T for the binding site.

6.2 Methods

Fish

Plasma from female black bream from a prior stress experiment (Haddy and Pankhurst, 1999) was used to investigate the effects of capture and confinement on SBP characteristics. In brief, black bream were captured from the wild by rod and line. A blood sample was taken within 5 min of the fish being hooked for all but 1 fish which was sampled 15 min after being hooked, but had an initial cortisol level below the assay detection limit. The fish were then finclipped and maintained in a 400L tank with aeration for 1, 6 or 24 h, until a second blood sample was taken. A subset of 5 fish from each time period was used in the current study.

Rainbow trout (2+ years old) maintained at the aquatic centre at the University of Tasmania as described by Pankhurst and Thomas (1998) were used for a subsequent confinement experiment. Five fish were netted into anaesthetic (2-phenoxyethanol) and blood sampled within 5 min of the first disturbance of the stock tank. After sampling, the fish were finclipped for identification and placed in a 400L covered tank, with aeration. The fish were anaesthetised and blood sampled again following 5 h of confinement with sporadic disturbance. Plasma from this experiment was assayed immediately without frozen storage.

To determine whether increased plasma cortisol (above the levels reached in our stressed fish) might affect SBP characteristics in trout, plasma from a prior stress experiment (Pankhurst and Van Der Kraak, 1999) was assayed. In this experiment, rainbow trout (2+ years old) were injected with cortisol at a dose of 5 $mg.kg^{-1}$ and serial blood samples were taken post-injection. Control fish were injected with saline. Plasma from control and cortisol-injected fish at 3 and 6 h post-injection was assayed. As the plasma had been assayed previously, a complete set of plasma was not available for the current study. Plasma from the same 5 saline-injected fish was available at both 3 and 6 h post-injection. However, plasma from only 4 cortisol-injected fish was available for both 3 and 6 h time periods. Therefore a different fish was used at 3 and 6 h to increase the sample size to 5 for each time period. No plasma was available from the 0 h samples for investigation of SBP.

Blood was stored on ice until centrifugation (15000 g for trout and 10000 g for bream blood) at 4 °C for 3 min. Plasma was stored at -20 °C until assay unless otherwise stated.

Binding Assay

The charcoal adsorption binding assay has been previously described (Hobby and Pankhurst, 1997). In brief, plasma with endogenous steroids stripped by an equal volume of dextran-coated charcoal (DCC; 20 $mg.ml^{-1}$) was diluted (100-fold for bream plasma and 200-fold for trout plasma) in phosphate buffer and incubated with a range of labelled E_2 ([2,4-^{3}H]estradiol (Sigma)) concentrations (1-20 nM for bream plasma and plasma from trout confined for 5 hours, and 0.05-20 nM for trout plasma from the cortisol injection experiment), both with and without 100-fold excess unlabelled E_2 as competitor. Tubes were equilibrated for 30 min at room temperature and overnight at 4 °C. The incubation was terminated by addition of 0.5 ml DCC (10 $mg.ml^{-1}$). Tubes were vortexed and incubated at 4 °C for 5 min prior to centrifuging at 2060 g and 4 °C for 10 min. The supernatant was decanted, 5 ml of Ecolite scintillation cocktail (ICN Biochemicals) added and vials were shaken and counted. Specific binding was calculated by subtracting non-specific binding from total binding. Estimates of the dissociation constant (k_D) as a measure of binding affinity, and the binding capacity (B_{max}) were determined using the computer programs EBDA (McPherson, 1985) and LIGAND (Munson and Rodbard, 1980).

To measure the relative affinity of SBP for cortisol, DCC-stripped, diluted pools of plasma from 3 or more fish were incubated with a constant amount (5 nM) of ^{3}H-E_2, and increasing concentrations (0 - 50 μM) of cortisol. The remainder of the assay protocol was as described above.

Radioimmunoassay

The protocol and reagents used for measurement of E_2, T and cortisol by radioimmunoassay (RIA) were as described in Pankhurst and Carragher (1992).

Statistics

Estimates of k_D and B_{max} calculated by LIGAND are displayed as geometric means with the associated standard error (se) on the estimate. Differences in SBP binding characteristics and plasma steroids between fish sampled before and after stress were determined using the paired t-test on estimates of k_D and B_{max} calculated in LIGAND from individual assays.

Differences in binding characteristics between trout injected with saline (controls) or cortisol (treated) were determined using LIGAND. Estimates of k_D and B_{max} were determined by simultaneously analysing all assays for control fish or cortisol-treated fish in LIGAND. Overlapping 95 % confidence limits on these estimates between control and cortisol-treated fish at a particular time were determined not to be significantly different. Differences in steroid levels between trout injected with cortisol or saline were determined using one-way ANOVA. Non-detectable levels of steroids were treated as being at the limits of detection for the statistical analyses. A significance level of 0.05 was used for all tests.

6.3 Results

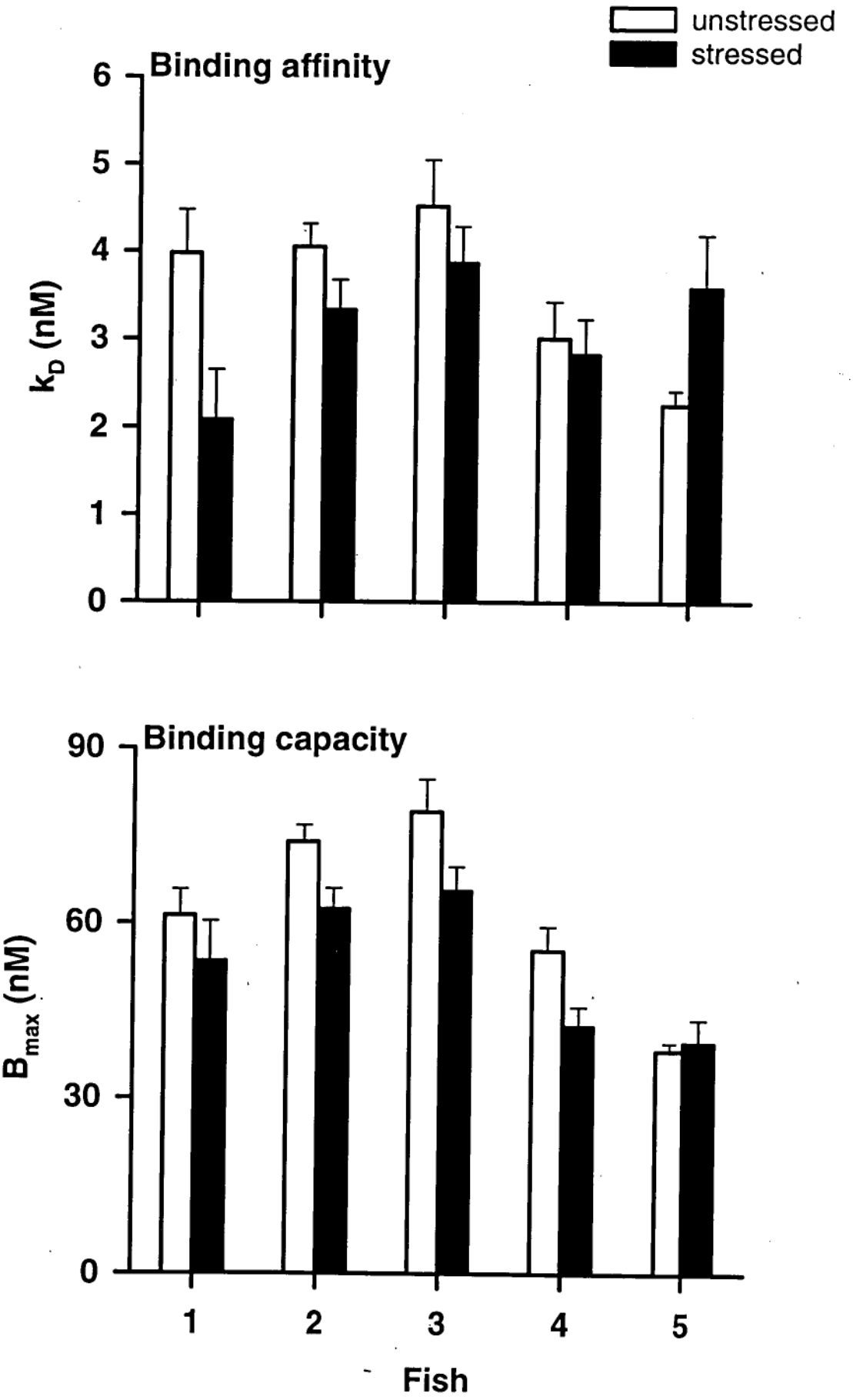
Changes in cortisol, E_2 and T with stress in black bream are shown in Table 6.1. Briefly, cortisol was higher after 1, 6 and 24 of confinement than at capture, while E_2 and T were significantly lower than at capture, in fish that had been confined for 6 or 24 h. Plasma from black bream that had been subject to capture followed by confinement for 6 h was assayed initially. The binding affinity (k_D) showed a tendency (in 4 of 5 fish) to be lower (ie. stronger binding) after stress, but the mean difference was not significant (mean difference $\pm$ se = 0.42 $\pm$ 0.52 nM, p = 0.4624) (Fig. 6.1). The number of binding sites (B_{max}) was significantly lower after stress (mean difference = 8.8 $\pm$ 2.7 nM, p = 0.0322,) when analysed by paired t-test (Fig. 6.1). The B_{max} in 4 of the fish was reduced by 13-23% of the initial measurement. In contrast, no significant differences in k_D or B_{max} were detected in plasma from black bream confined for 1 h or 24 h after capture, nor were any non-significant trends evident from the samples at these times (data not shown).

Plasma cortisol concentrations in rainbow trout were below 10 $ng.ml^{-1}$ in all fish at the start of the experiment and significantly increased after 5 h of confinement and disturbance

Table 6.1: Plasma levels of cortisol, E_2 and T ($ng.ml^{-1}$) in black bream at capture, after confinement, and the mean difference at 1, 6 or 24 h after capture. Values are mean ± se, n = 5. Significant differences by paired t-test are shown by *. (DL indicates that steroid levels of all fish in the group were at or below the assay detection limit).

		At capture	After confinement		Difference
Cortisol	1 h	0.7 ± 0.2	41.6 ± 4.9	+	40.9 ± 4.8 *
	6 h	0.3 (DL)	18.5 ± 5.0	+	13.1 ± 4.5 *
	24 h	1.0 ± 0.4	21.7 ± 1.9	+	20.7 ± 2.0 *
E_2	1 h	5.2 ± 0.9	3.5 ± 0.6	-	1.7 ± 0.7
	6 h	4.7 ± 0.9	2.2 ± 0.8	-	2.7 ± 0.7 *
	24 h	7.6 ± 2.5	1.2 ± 0.5	-	6.4 ± 2.2 *
T	1 h	8.8 ± 2.5	2.8 ± 0.3	-	5.9 ± 2.4
	6 h	4.4 ± 0.6	1.1 ± 0.3	-	3.6 ± 0.4 *
	24 h	2.7 ± 0.6	0.7 ± 0.1	-	1.9 ± 0.7 *

Figure 6.1: Binding affinity and capacity of SBP for E_2, before and after 6 h confinement in black bream. Estimates of k_D and B_{max} are shown for each fish at capture and after stress, with se on these estimates indicating a measure of precision, shown as capped bars.


unstressed
stressed
Binding affinity
k_D (nM)
0
1
2
3
4
5
6
Binding capacity
B_{max} (nM)
0
30
60
90
1
2
3
4
5
Fish

(mean difference = 72.4 ± 15.9 $ng.ml^{-1}$, p = 0.0105) (Table 6.2). However, no differences in either k_D or B_{max} were evident after 5 h of confinement stress (Fig. 6.2). Fish thought to be mid-vitellogenic females were selected for the experiment, however initial plasma levels of E_2 and T were low, and the majority of the stock were found to be only in the early stages of vitellogenesis. Plasma T, however, decreased significantly following confinement (mean difference = 2.1 ± 0.3 $ng.ml^{-1}$, p = 0.0024). Levels of E_2 were low and did not change following stress.

Confinement stress had no effect on k_D or B_{max} in trout, but cortisol levels, although elevated with stress, were well below peak levels reached in this species. Therefore, the effect of artificial elevation of plasma cortisol on plasma SBP characteristics was investigated. Steroid levels at the start of the experiment did not differ between the saline and cortisol-injected fish (Table 6.3). Cortisol levels were significantly elevated in the fish injected with cortisol compared to control fish at 3 and 6 h. Plasma E_2 was depressed in the cortisol-injected fish relative to the controls at 3 and 6 hours post-injection and plasma T was depressed at 3 hours post-injection in the cortisol-injected fish. There were no significant differences or apparent trends in k_D or B_{max} at 3 h (Fig. 6.3). Trout that had been injected with cortisol tended to have lower B_{max} and k_D than saline-injected fish at 6 h post-injection (Fig. 6.3), however, these trends were not statistically significant (95 % CL for B_{max} = 105-167 and 72-109 nM, and 95 % CL for k_D = 0.92-1.85 and 0.70-1.36 nM, for control and cortisol injected fish respectively).

There was some competition by cortisol with E_2 for the binding site on bream SBP (Fig. 6.4). A cortisol concentration of 100 times that of ^3H-E_2 could displace nearly 30% of the bound ^3H-E_2. However, the relative affinity for cortisol binding compared to E_2 binding was still very low (Table 6.4). There was less competition of cortisol with E_2 for trout SBP binding sites over the range of relative competitor concentrations investigated (up to 10 000 fold excess). Even at a cortisol concentration of 10 000 times the concentration of ^3H-E_2, only about 10% of the ^3H-E_2 bound to trout SBP was displaced (Fig. 6.4, Table 6.4).

Table 6.2: Plasma levels of cortisol, E_2 and T ($ng.ml^{-1}$) in rainbow trout before and after confinement, and the mean difference following 5 h confinement. Values are mean ± se, n = 5. Significant differences by paired t-test are shown by *.

	Before confinement	After confinement		Difference
Cortisol	5.8 ± 1.2	78.2 ± 15.5	+	72.4 ± 15.9 *
E_2	1.0 ± 0.3	1.0 ± 0.2	-	0.0 ± 0.0
T	3.7 ± 0.5	1.6 ± 0.2	-	2.1 ± 0.3 *

Figure 6.2: Binding affinity and capacity of SBP for E_2, before and after 5 h confinement in rainbow trout. Details as for Fig. 6.1.

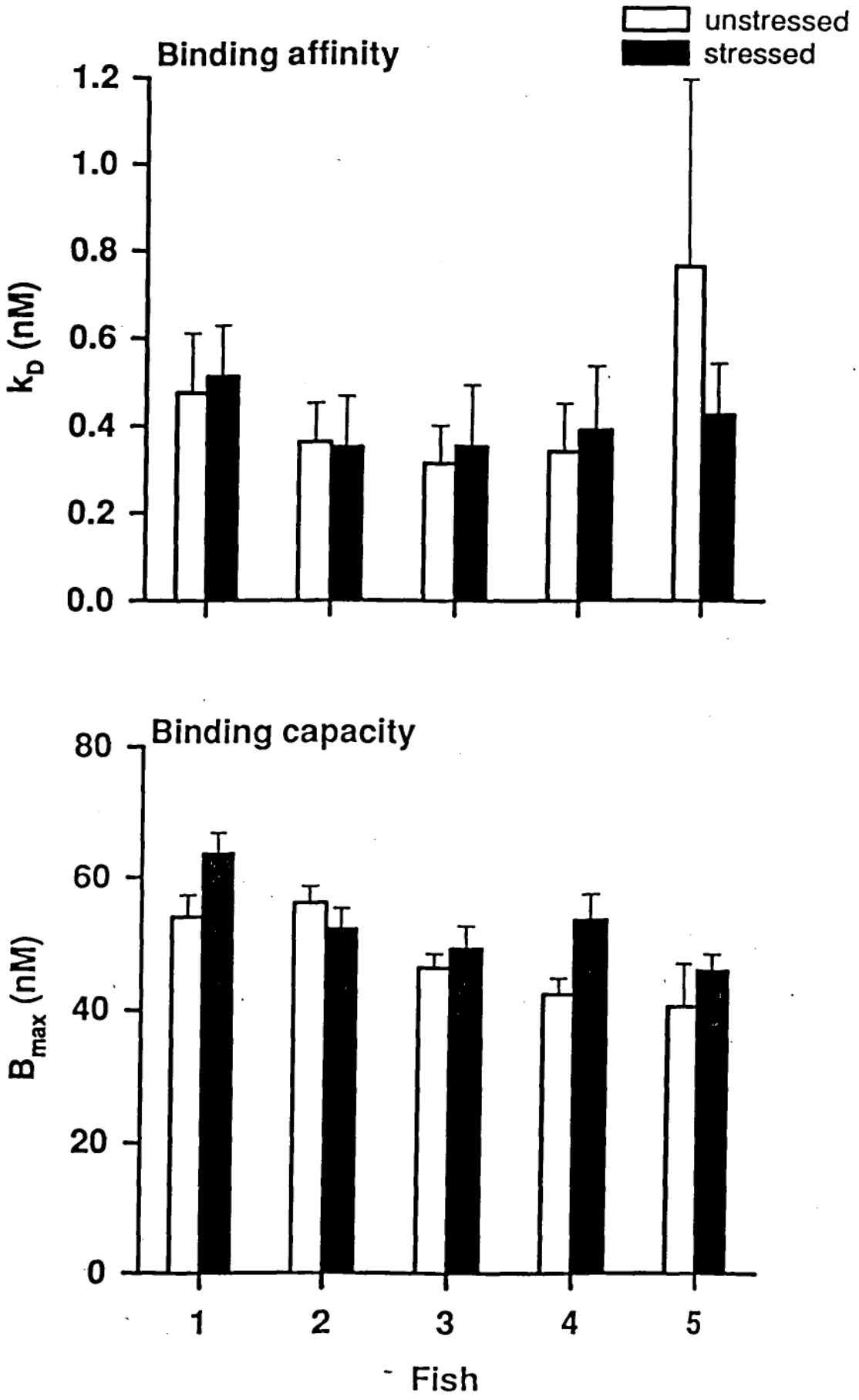

unstressed
stressed
Binding affinity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
k_D (nM)
Binding capacity
80
60
40
20
0
B_{max} (nM)
1
2
3
4
5
Fish

Table 6.3: Plasma levels of cortisol, E_2 and T ($ng.ml^{-1}$) in trout injected with either 5 $mg.kg^{-1}$ cortisol or saline at the beginning of the experiment and 3 and 6 h post-injection. Values are mean ± se (n = 5 for all groups, except n = 6 for cortisol injected fish at 0 h). Significant differences between saline and cortisol treated fish at each time period by 1-way ANOVA are shown by *.

	Cortisol		E_2		T	
	Saline	Cortisol	Saline	Cortisol	Saline	Cortisol
0 h	33.9 ± 10.6	29.6 ± 9.3	13.8 ± 1.3	10.3 ± 2.3	8.0 ± 0.4	7.3 ± 0.6
3 h	47.1 ± 4.3 *	112.7 ± 26.8	13.9 ± 1.8 *	6.5 ± 2.0	6.6 ± 1.3 *	2.0 ± 0.6
6 h	41.5 ± 3.2 *	67.7 ± 5.9	12.8 ± 1.8 *	5.0 ± 1.3	6.0 ± 1.1	5.5 ± 1.7

Table 6.4: Binding characteristics for E_2 and cortisol binding to SBP, and affinity of binding relative to E_2. Assays from bream at capture from the 1 h and 6 h groups, and trout at the start of the confinement experiment were used for the estimates of k_D and B_{max} for E_2.

	Steroid	k_D (nM)	95 % CL	Maximum inhibition of binding relative to E_2 (mean ± se)	Affinity relative to E_2	n
bream	E_2	3.47	(2.89, 4.16)	100	100	10
	cortisol	1996	(981, 4059)	87.5 ± 0.33	0.2	2
trout	E_2	0.41	(0.24, 0.70)	100	100	5
	cortisol	_	_	10.2 ± 3.87	<0.01	2

Figure 6.3: Binding affinity and capacity of SBP for E_2 in rainbow trout at 3 and 6 h after injection with either 5 mg.kg-1 cortisol or saline. Data are mean ± se (n = 5).

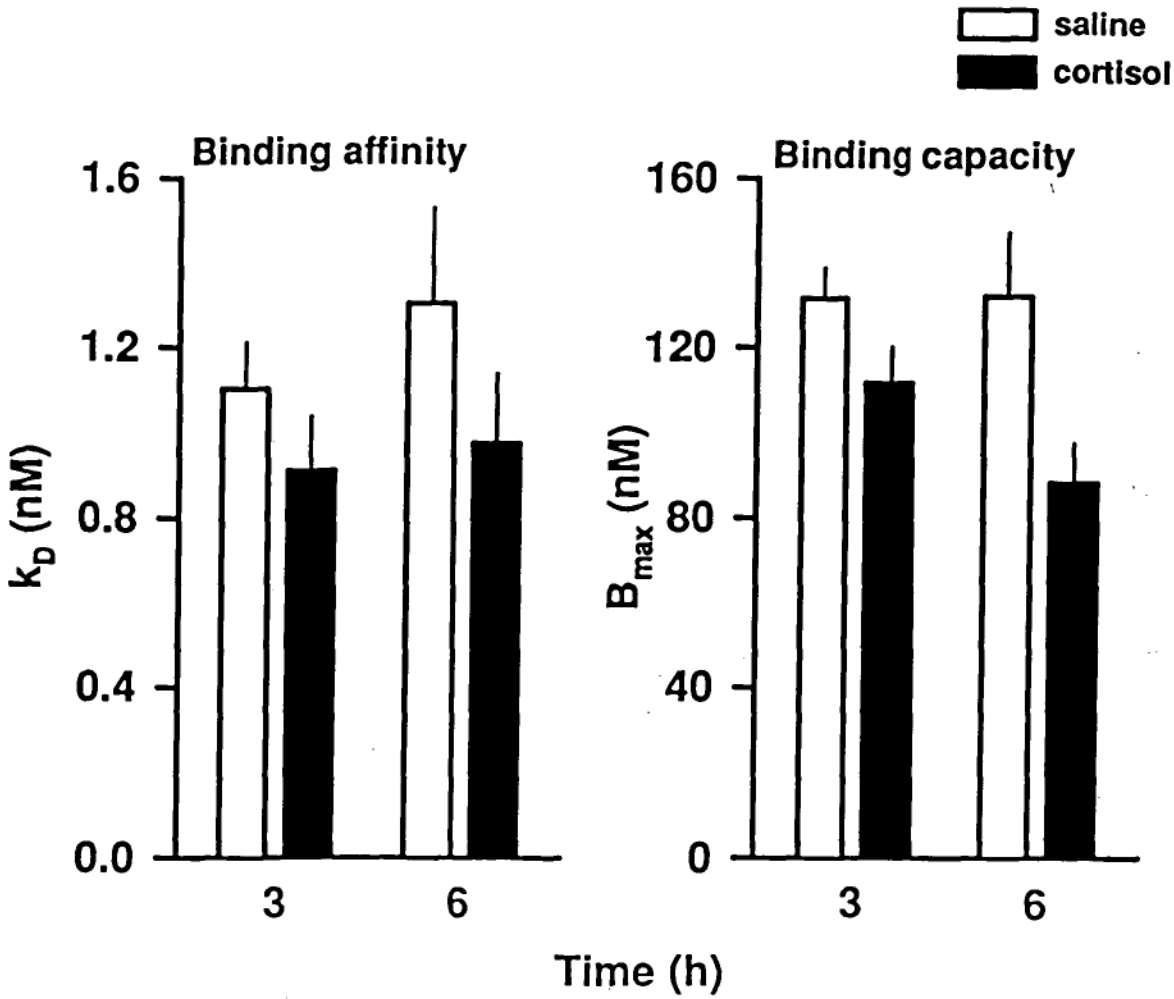
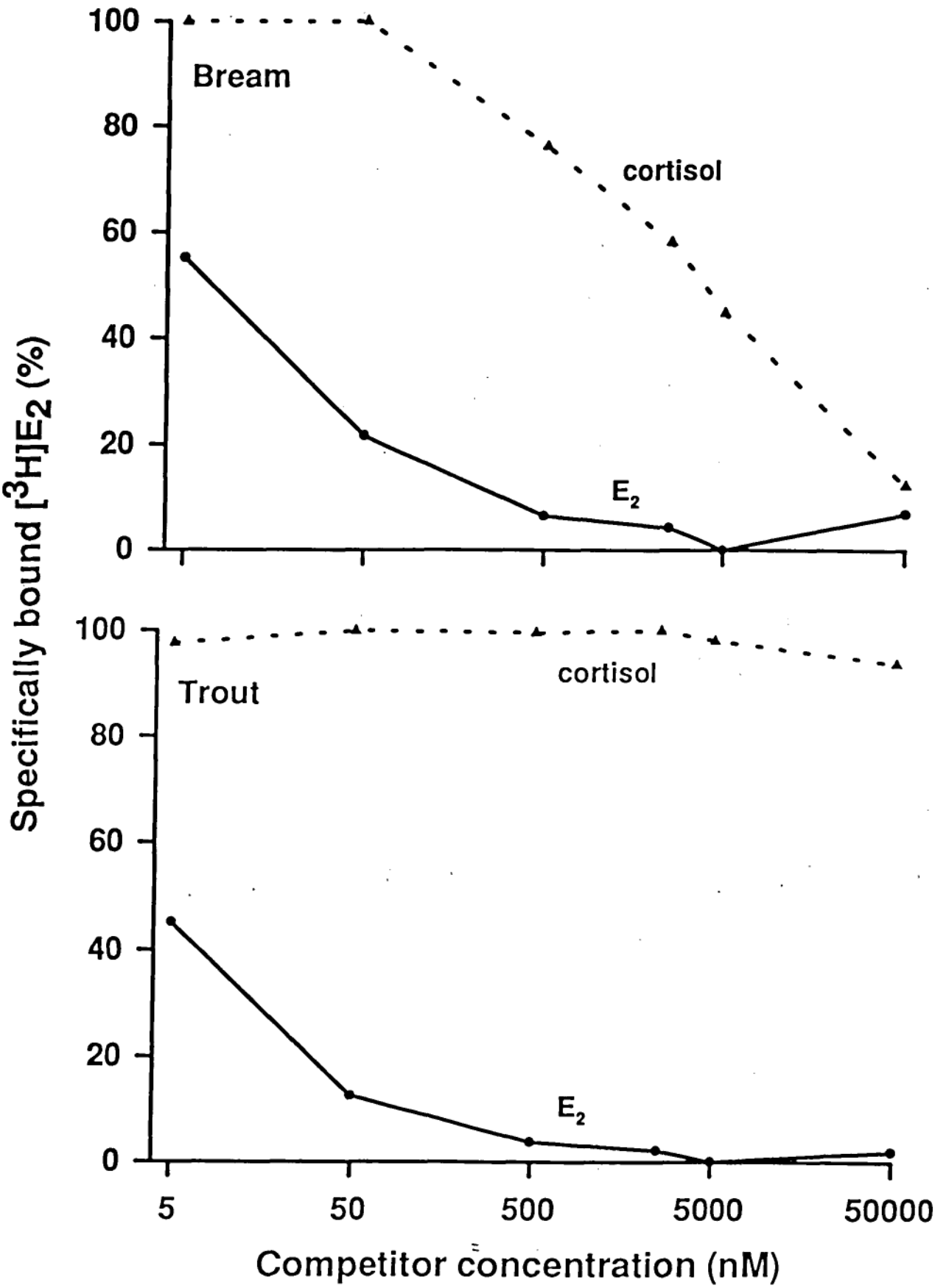

saline
cortisol
Binding affinity
Binding capacity
k_D (nM)
B_{max} (nM)
1.6
1.2
0.8
0.4
0.0
160
120
80
40
0
3
6
3
6
Time (h)

Figure 6.4: Representative competitive binding curves for E_2 and cortisol with SBP for rainbow trout and black bream.


Bream
cortisol
E_2
Trout
cortisol
E_2
100
80
60
40
20
0
5
50
500
5000
50000
Specifically bound [^{3}H]E_2 (%)
Competitor concentration (nM)

6.4 Discussion

The binding capacity of black bream plasma was significantly lower after 6 hours of confinement than at capture, but confinement for 1 or 24 hours did not alter binding capacity. There are several possible explanations for this. The reduction in binding capacity may occur between 1 and 6 hours, and may be only temporary so that binding capacity has recovered to pre-stress levels somewhere between 6 and 24 hours post-capture. Alternatively, the presence or absence of a stress effect on SBP may be influenced by the time of capture of the fish for the various treatment groups. Bream for the 6 hour confinement group were captured between 0520 and 0650, with the second blood sample taken around midday. However, fish for the 1 and 24 hour confinement groups were all captured after midday (1340 - 1830 for the 1 h group and 1800 - 2140 for the 24 h group). There is a diurnal pattern in plasma T but not E_2 in black bream associated with daily cycles of oocyte recruitment and ovulation (Haddy and Pankhurst, 1998). It is possible that binding capacity in this species also varies in a diurnal manner, and that SBP may be more susceptible to stress-induced alterations at certain parts of the cycle. SBP binding capacity in humans varies on a diurnal basis (Myers *et al.*, 1986) as does corticosteroid binding globulin (Westphal, 1986). However, the binding capacity of fish caught at different times of the day did not differ (95 % CL = 50-70 and 48-60 nM for fish caught in the morning and afternoon or evening respectively).

There were no changes in SBP binding characteristics after 5 h confinement of rainbow trout. Peak cortisol levels of rainbow trout from the University of Tasmania stock are not as high as levels recorded for other rainbow trout stocks. For example, plasma cortisol in our fish reaches a maximum of about 110 $ng.ml^{-1}$ after 1 h of confinement (Thomas *et al.*, 1999), compared to levels of 200 - 900 $ng.ml^{-1}$ reported for some rainbow trout populations after stress (Barton and Iwama, 1991). The reduction in E_2 and T seen in response to stress in many non-salmonids (Pankhurst and Van Der Kraak, 1997) and in wild rainbow trout (Pankhurst and Dedual, 1994) is also absent or inconsistent in our trout (Pankhurst, 1998) (N. Pankhurst unpublished data). Other domestic stocks of rainbow trout also fail to show a consistent suppression of E_2 and T by stress (Campbell *et al.*, 1994). The absence of a stress effect on rainbow trout SBP after 5 h confinement stress may be because peak cortisol levels are towards the low end of the range for stressed trout. This is supported by

the depression in B_{max} seen in cortisol-injected trout at 6 h post-injection. While the effect was not statistically significant, the marked reduction of more than 30% may be of biological importance. It is also possible that the depression of B_{max} may be partially masked by the relatively high plasma cortisol levels of the fish at the start of the experiment.

Estimates of k_D and B_{max} from trout confined for 5 h were lower than from cortisol or saline-treated fish. Both experiments were carried out at the end of March (during the period of vitellogenesis) but in different years. The fish injected with cortisol or saline were late vitellogenic females, whereas the trout population from which the fish were confined for 5 h were only in the early stages of vitellogenesis. In a companion study, we have found that B_{max} is higher in vitellogenic than non-reproductive trout (Chapter 5, this volume). Our assay protocol for trout was also changed between determination of SBP from confined fish and injected fish. In the early protocol, there were no ^{3}H-E_2 concentrations below the calculated k_D which suggested the k_D value might not be reliable. For subsequent determinations of SBP in trout, 4 lower ^{3}H-E_2 concentrations were added to the assay. We believe it is unlikely that the higher values for k_D and B_{max} from the injected fish resulted from the changed assay protocol. We found very little difference in binding parameters from the same fish determined with both assay protocols (A. Hobby and N. Pankhurst unpublished data). Plasma from the injected fish had also been stored, and thawed and refrozen at least twice for steroid determination, whereas plasma from the confined fish was assayed fresh. Our unpublished data, however, indicated that k_D and B_{max} calculated from assays of fresh plasma did not differ from binding characteristics calculated from the same plasma frozen for between 1 week and 6 months for trout or black bream. Nor was there a difference between trout plasma assayed fresh, or frozen and thawed twice. In other species, some loss of binding activity of dogfish (*Scyliorhinus canicula*), haddock (*Melanogrammus aeglefinus*) and skate (*Raja radiata*) SBP has been found with storage at -17 to -18 °C (Martin, B., 1975; Idler and Freeman, 1973), although proteins binding cortisol were more affected than those binding T (Idler and Freeman, 1973). Binding in plasma from Atlantic salmon (*Salmo salar*) remained stable at -17 °C (Idler and Freeman, 1973) and human SBP also remains stable in serum samples after repeated freezing and thawing but storage of highly purified SBP reduces activity (Lindstedt *et al.*, 1985). Regardless of this, plasma from both saline and cortisol-injected

fish was treated the same way so the relative differences, or lack of them, remain. It appears more likely that the differences in k_D and B_{max} from the two experiments result primarily from the different stages of maturity of the respective groups of fish.

The apparent reduction of B_{max} by 30% seen in trout appears to be an effect of cortisol rather than a downstream effect of some other stress-induced change. We were unable to determine whether the reduction of B_{max} seen in bream plasma after 6 h of confinement was an effect of cortisol or not. Cortisol levels of wild bream maintained in captivity with no handling are similar to basal levels of wild-caught fish. However, cortisol levels increase rapidly with handling and remain high (J. Haddy and N. Pankhurst unpublished data), such that it is essentially impossible to distinguish effects due to handling, from effects resulting from cortisol treatment.

The reduction in binding capacity shown in trout 6 h after cortisol injection is in contrast to the only other study investigating the effect of cortisol on plasma E_2 binding capacity in fish. Pottinger and Pickering (1990) measured a 33% increase in binding capacity in immature female rainbow trout 2 weeks after cortisol implantation, and the effect was lost by 4 weeks post-implant. The results from Pottinger and Pickering (1990) and our study, however, are not mutually exclusive. In the current study, we were investigating the possibility of a rapid response (hours) of SBP to stress. Pottinger and Pickering (1990) measured SBP levels as part of a study of the effect of cortisol levels on hepatic E_2 receptor abundance over a time frame of weeks. It is possible that the initial decrease in plasma SBP levels is followed by a slower increase above initial levels that is apparent at 2 weeks, and a subsequent return to normal sometime between 2 and 4 weeks later. Pottinger and Pickering (1990) also suggested the decline in plasma SBP binding capacity at 4 weeks may have been due to a decline in the efficiency of the implant at later stages of the experiment.

If the decreases in B_{max} after 6 hours confinement of black bream and 6 h after cortisol injection in rainbow trout, represent a typical response to stress, then there are several implications for reproduction. The most obvious is that if there are less available SBP binding sites, then a smaller proportion of circulating E_2 and T is protected from metabolism. The equilibrium between free and bound steroid depends on the concentration

of steroid, the binding capacity and affinity of the SBP, and the binding affinity and capacity of any other substances in the blood that also bind steroids. Steroid circulates bound to albumin or an albumin-like protein as well as SBP, in the plasma of many animals (Siiteri *et al.*, 1982). The affinity of albumin for steroids is low ($k_D \sim 10^{-4}$ - 10^{-5} M for E_2; Clark and Peck, 1977), but the capacity of albumin for ligand is very large (4 - 6^{-4} M in most animals; Sandberg, 1977). This means that in humans for example, 30 - 50 % of circulating T and 60 - 80 % of E_2 is bound to albumin (Dunn *et al.*, 1981), and 40 - 80 % of SBP may be unliganded (Hryb *et al.*, 1990). The high concentration of SBP in bream relative to peak steroid levels may be necessary to compete with albumin or an albumin-like protein to protect the minimum amount of steroid needed for reproduction. Binding of E_2 to human SBP appears to be non-linear (Zeginiadou *et al.*, 1997). At very low SBP concentrations, most E_2 is bound to albumin, but once the concentration of SBP is over the threshold, a small increase in the concentration of SBP can result in a disproportionately large increase in the amount of steroid bound to SBP. If the same scenario is present in fish, it is possible that a small reduction in B_{max} may substantially reduce the amount of SBP-bound steroid. In this way, the lower B_{max} with stress may be another factor contributing to the drop in plasma E_2 and T levels.

The binding affinity of black bream SBP also showed a tendency to change in the group of fish confined for 6 hours after capture. The k_D appeared lower in 3 fish, higher in 1 fish and unchanged in 1 fish. This tendency was also present in rainbow trout 6 h after cortisol injection compared to saline-injected controls at the same time. The non-significant trend towards stronger binding after stress might be a compensation for the decline in B_{max}. This also suggests the possibility of lower steroid levels reaching the receptors if steroid is slower to dissociate from SBP as a result of lower k_D. Increased plasma concentrations of free fatty acids have been shown in response to stressors in some mammals (Boonstra and Tinnikov, 1998), and this in turn has been related to inhibition of binding of ligands to SBP (Martin *et al.*, 1986). It is not known whether plasma free fatty acid levels are related to stress in lower vertebrates, however, polyunsaturated nonesterified fatty acids inhibit binding by reducing the affinity of SBP for steroids in goldfish (*Carassius auratus*) (Van Der Kraak and Biddiscombe, 1999).

Displacement of ^{3}H-E_2 from SBP at a range of cortisol concentrations showed that cortisol did not compete very effectively with E_2 for the SBP binding site in bream at concentrations of less than 10 times the concentration of E_2. However, at cortisol concentrations of 100 times the concentration of E_2, between 20 and 30 % of the bound E_2 was displaced. Cortisol levels for the bream in our study were around 40 ng.ml^{-1} after 1h stress, when E_2 levels were about 4 ng.ml^{-1}. After 24 h of stress, plasma cortisol levels were about 20 times that of circulating E_2 (cortisol ~ 20 ng.ml^{-1}, E_2 ~ 1 ng.ml^{-1}). At these relative concentrations of steroids, there may be some displacement of E_2 from SBP by cortisol. However, cortisol levels in excess of 200 ng.ml^{-1} have been recorded in wild bream after capture, handling and transport (Haddy and Pankhurst unpublished data) when plasma E_2 levels in the same fish are at the assay detection limit of 0.2 ng.ml^{-1}. In this case the cortisol concentration is about 1000 times that of E_2, and under these conditions, cortisol has the ability to displace about 50% of bound E_2. It is possible that relative differences in steroid concentrations of this magnitude act to push the remaining E_2 from SBP into the circulating free pool and towards metabolism, further decreasing the concentration of E_2 in the plasma. In contrast, E_2 bound to rainbow trout SBP was not displaced by physiological levels of cortisol. This finding is in agreement with the study on rainbow trout by Pottinger and Pickering (1990) where there was no displacement of E_2 by cortisol at concentrations up to 1000 times that of E_2.

The changes in SBP observed in the present study, and the competition of physiological concentrations of cortisol for SBP binding sites may generate a component of the stress-induced falls in plasma levels of E_2 reported across a range of species.

6.5 References

Barton, B.A., and Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1**, 3-26.

Boonstra, R., and Tinnikov, A.A. (1998). Increased corticosteroid binding capacity of plasma albumin but not of corticosteroid-binding globulin caused by ACTH-induced changes in free fatty acid concentrations in snowshoe hares and rabbits. *J. Endocr.* **156**, 205-212.

Brooks, S., Pottinger, T.G., Tyler, C.R., and Sumpter, J.P. (1995). Does cortisol influence egg quality in the rainbow trout, *Oncorhynchus mykiss*? *In* "Reproductive Physiology of Fish 1995" (F.W. Goetz, and P. Thomas, Eds.), pp. 180. FishSymp95, Austin, Texas.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biol. Reprod.* **47**, 1140-1150.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* **120**, 151-169.

Carragher, J.F., and Pankhurst, N.W. (1991). Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). *In* "Reproductive Physiology of Fish 1991" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 253-255. FishSymp91, Sheffield.

Carragher, J.F., and Sumpter, J.P. (1990). The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *Gen. Comp. Endocrinol.* **77**, 403-407.

Carragher, J.F., Sumpter, J.P., Pottinger, T.G., and Pickering, A.D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *Gen. Comp. Endocrinol.* **76**, 310-321.

Clark, J.H., and Peck, E.J. (1977). Steroid hormone receptors: Basic principles and measurement. *In* "Receptors and Hormone Action" (B.W. O'Malley, and L. Birmbaumer, Eds.), pp. 383-410. Academic Press, New York.

Clearwater, S.J., and Pankhurst, N.W. (1997). The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. *J. Fish Biol.* **50**, 429-441.

Dunn, J.F., Nisula, B.C., and Rodbard, D. (1981). Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* **53**, 58-68.

Haddy, J.A., and Pankhurst, N.W. (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Mar. Freshwater Res.* **49**, 389-397.

Haddy, J.A., and Pankhurst, N.W. (1999). Stress-induced changes in concentrations of plasma sex steroids in black bream, *Acanthopagrus butcheri. J. Fish Biol.* **55**, 1304-1316.

Hammond, G.L. (1990). Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocr. Rev.* **11**, 65-79.

Hobby, A.C., and Pankhurst, N.W. (1997). Comparative aspects of steroid binding protein (SBP) in four fishes: a salmonid, a pleuronectid and two sparids. *In* "Advances in Comparative Endocrinology" (S. Kawashima, and S. Kikuyama, Eds.), pp. 1369-1373. Monduzzi Editore, Bologna.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048-6054.

Hsueh, A.J.W., Billig, H., and Tsafriri, A. (1994). Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr. Rev.* **15**, 707-724.

Idler, D.R., and Freeman, H.C. (1973). Stability to freezing of steroid binding proteins in fish plasma. *Comp. Biochem. Physiol.* **44B**, 179-183.

Joseph, D.R. (1994). STructure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197-280.

Lindstedt, G., Lundberg, P., Hammond, G.L., and Vihko, R. (1985). Sex hormone-binding globulin - still many questions. *Scand. J. Clin. Lab. Invest.* **45**, 1-6.

Martin, B. (1975). Steroid-protein interactions in nonmammalian vertebrates. I. Elasmobranch steroids-binding protein (E.SBP) in dogfish serum (*Scyliorhinus canicula*). *Gen. Comp. Endocrinol.* **25**, 42-51.

Martin, M.E., Vranckx, R., Benassayag, C., and Nunez, E.A. (1986). Modifications of the properties of human sex steroid-binding protein by nonesterified fatty acids. *J. Biol. Chem.* **261**, 2954-2959.

McPherson, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacological Methods* **14**, 213-228.

Munson, P.J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.

Myers, J.S., Matej, L.A., Bremner, W.J., and Plymate, S.R. (1986). Diurnal and age related changes in testosterone, sex-hormone-binding globulin, and calculated free testosterone. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 634. John Libbey Eurotext, London.

Pankhurst, N.W. (1998). Further evidence of the equivocal effects of cortisol on *in vitro* steroidogenesis by ovarian follicles of rainbow trout *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **19**, 315-323.

Pankhurst, N.W., and Carragher, J.F. (1992). Oocyte maturation and changes in plasma steroid levels in snapper *Pagrus* (=*Chrysophrys*) *auratus* (Sparidae) following treatment with human chorionic gonadotropin. *Aquaculture* **101**, 337-347.

Pankhurst, N.W., and Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *J. Fish Biol.* **45**, 1013-1025.

Pankhurst, N.W., and Thomas, P.M. (1998). Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. *Aquaculture* **166**, 163-177.

Pankhurst, N.W., and Van Der Kraak, G. (1997). Effects of stress on reproduction and growth of fish. *In* "Fish Stress and Health in Aquaculture" (G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, Eds.), pp. 73-93. Cambridge University Press, Cambridge.

Pankhurst, N.W., and Van Der Kraak, G. (2000) Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout *in vivo*, through the action of cortisol. *Gen. Comp. Endocrinol.* in press.

Pankhurst, N.W., Van Der Kraak, G., and Peter, R.E. (1995a). A reassessment of the inhibitory effects of cortisol on ovarian steroidogenesis. *In* "Reproductive

Physiology of Fish" (F.W. Goetz, and P. Thomas, Eds.), pp. 195. FishSymp 95, Austin, Texas.

Pankhurst, N.W., Van Der Kraak, G., and Peter, R.E. (1995b). Evidence that the inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. *Gen. Comp. Endocrinol.* **99**, 249-257.

Pottinger, T.G., and Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **80**, 264-273.

Sandberg, A.A. (1977). Regulation of plasma steroid binding proteins. *In* "Endocrinology" (V.H.T. James, Ed.), pp. 452-457. Excerpta Medica, Amsterdam-Oxford.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., and Kuhn, R.W. (1982). The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38**, 457-510.

Sumpter, J.P., Pottinger, T.G., Rand-Weaver, M., and Campbell, P.M. (1994). The wide-ranging effects of stress of fish. *In* "Perspectives in Comparative Endocrinology" (K.G. Davey, R.E. Peter, and S.S. Tobe, Eds.), pp. 535-538. National Research Council of Canada, Ottawa.

Thomas, P.M., Pankhurst, N.W., and Bremner, H.A. (1999). The effect of stress and exercise on post-mortem biochemistry of Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*. *J. Fish Biol.* **54**, 1177-1196.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

Westphal, U. (1986). "Steroid-Protein Interactions II", p. 592. Monographs on Endocrinology 27, Springer-Verlag, New York.

Zeginiadou, T., Kolias, S., Kouretas, D., and Antonoglou, O. (1997). Nonlinear binding of sex steroids to albumin and sex hormone binding globulin. *Eur. J. Drug Metab. Pharmacokinet.* **22**, 229-235.

7. General Discussion

7. General Discussion

Studies of SBP function have focused predominantly on humans, driven by the need to understand the role of SBP in various endocrine disorders and endocrine-related cancers (Westphal, 1986). The majority of reports of SBP in lower vertebrates describe the presence and binding characteristics of SBP, but studies of factors affecting SBP are fewer (eg. Foucher *et al.*, 1991; Foucher *et al.*, 1992; Van Der Kraak and Biddiscombe, 1999). The rationale behind the present study was to use a comparative approach to investigate various aspects of SBP in teleost fish with the aim of furthering the understanding of the role of SBP in teleost reproduction. Specific binding of E_2 and/or T to plasma proteins has been shown in more than 40 species of teleost fishes (eg. Fostier and Breton, 1975; Laidley and Thomas, 1994; Pasmanik and Callard, 1986; Chang and Chen, 1990a). Binding characteristics for binding to SBP have been measured in fewer species. Estimates of the binding affinity (k_D) range from as low as 2 nM for E_2 and T in the goldfish *Carassius auratus* (Pasmanik and Callard, 1986; Van Der Kraak and Biddiscombe, 1999) to 48 nM for E_2 in the brown trout *Salmo trutta* (Pottinger, 1986). Measurements of the binding capacity (B_{max}) vary from around 1 nM in the common carp *Cyprinus carpio* (Chang and Chen, 1990b) to 2000 nM in the rainbow trout *Salmo gairdneri* = *Oncorhynchus mykiss* (Fostier and Breton, 1975), but more commonly range from 100 to 500 nM (Pottinger, 1988; Foucher *et al.*, 1992; Laidley and Thomas, 1994; Van Der Kraak and Biddiscombe, 1999).

The variability in SBP binding characteristics in fishes may have a phylogenetic basis. There is a general trend for decreased B_{max} in more phylogenetically 'modern' vertebrates (Callard and Callard, 1987). It is also possible that binding characteristics within the teleosts may be related to the mode of reproduction of the species, or even more simply, to the peak plasma levels of steroids present during reproduction. It was thought that an understanding of species differences in binding characteristics of SBP might help explain the importance of SBP in reproductive processes in fish. The present study was an attempt to address this, firstly by measurement of SBP binding characteristics in 4 different species (rainbow trout *Oncorhynchus mykiss*, black bream *Acanthopagrus butcheri*, snapper (= red sea bream) *Pagrus auratus*, and greenback flounder *Rhombosolea tapirina*), using the same method so species differences would not be confounded by differences in methods

(Chapter 3, this volume). It was also thought that differences in SBP binding characteristics between reproductive and non-reproductive fish from 3 different orders might clarify whether SBP has a single mode of action, or multiple modes of action depending on species (Chapter 5, this volume). Stress responses also differ between species (Pankhurst and Van Der Kraak, 1997). A rapid decline in plasma levels of E_2 and/or T is seen in many but not all species (Pankhurst and Van Der Kraak, 1997). It was thought that differences at this level of the stress response might be driven by stress or cortisol effects on SBP, and this possibility was investigated in Chapter 6 (this volume).

Optimisation of the methods for measuring SBP binding characteristics was a large component of the present study (Chapter 2, this volume). Problems with the DCC-adsorption method for separating SBP-bound and free steroid became apparent after investigation of dissociation of E_2 from SBP (Chapter 3, this volume). However, dissociation of the E_2-SBP complex during the 5 min incubation time was about 50 % and not up to 90 % as after 5 min of dissociation. Despite shortcomings of the DCC-adsorption method with a 5 min incubation time, this method is still valid for comparisons within a species as in Chapters 5 and 6. To confirm the reproducibility of results for each species obtained using DCC-adsorption, values of k_D and B_{max} from hot saturation (increasing amounts of both labelled and unlabelled E_2) and cold saturation assays (a constant concentration of ^{3}H-E_2 and increasing amounts of unlabelled competitor) from each chapter were compared. Estimates of both k_D and B_{max} for trout, bream and snapper were consistent. Estimates of k_D from 16 different set of assays for trout plasma ranged from 0.39 nM to 1.48 nM (Table 7.1). Values of B_{max} from the same assays ranged from 47 nM to 132 nM (Table 7.1). Estimates of k_D from 12 sets of assays of bream plasma varied only slightly from 2.84 nM to 3.97 nM, and the spread of B_{max} values was similarly tight, ranging from 33 nM to 60 nM (Table 7.2). Only 2 sets of assays were performed with snapper plasma, and the comparison between hot and cold saturation assays shows reasonable similarity (Table 7.3). However, there are substantial differences present from the various assays of flounder SBP (Table 7.4). Estimates of k_D varied from 77 nM to 239 nM, and B_{max} values also ranged from 96 nM to 407 nM (Table 7.4). These differences are most likely a result of the lower affinity binding and the extremely rapid dissociation rate

Table 7.1: Estimates of k_D and B_{max} and associated 95 % confidence limits from all assays of rainbow trout plasma. (F = cortisol).

Method	Sample	k_D (nM)	Lower 95% CL	Upper 95% CL	B_{max} (nM)	Lower 95% CL	Upper 95% CL	n
hot sat	Chapter 3	**0.44**	0.33	0.58	**92**	83	101	3 pools
cold sat	Chapter 4	**0.90**	0.43	1.87	**88**	68	114	7 (mix)
hot sat	vitellogenic [1]	**0.65**	0.54	0.79	**124**	115	134	5 fish
cold sat	vitellogenic [1]	**1.48**	0.90	2.44	**105**	87	127	5 fish
hot sat	non-reprod [1]	**0.39**	0.22	0.72	**77**	63	94	5 fish
cold sat	non-reprod [1]	**0.58**	0.25	1.35	**75**	56	102	5 fish
hot sat	males start [1] (control)	**0.61**	0.27	1.36	**50**	36	71	5 fish
hot sat	males start [1] (E_2 injected)	**0.67**	0.40	1.14	**58**	46	72	5 fish
hot sat	males end [1] (control)	**0.43**	0.28	0.68	**60**	51	71	3 fish
hot sat	males end [1] (E_2 injected)	**1.34**	0.85	2.12	**47**	37	60	5 fish
hot sat	unstressed [2]	**0.41**	0.24	0.70	**47.5**	42.8	52.7	5 fish
hot sat	stressed [2]	**0.41**	0.28	0.59	**52.9**	49.1	56.9	5 fish
hot sat	control 3 h [2]	**1.10**	0.90	1.34	**132**	118	147	5 fish
hot sat	F inj 3 h [2]	**0.91**	0.69	1.21	**112**	96	130	5 fish
hot sat	control 6 h [2]	**1.30**	0.91	1.85	**132**	104	167	5 fish
hot sat	F inj 6 h [2]	**0.98**	0.70	1.36	**88**	72	109	5 fish

[1] Chapter 5.

[2] Chapter 6.

Table 7.2: Estimates of k_D and B_{max} and associated 95 % confidence limits from all assays of black bream plasma.

Method	Sample	k_D (nM)	Lower 95% CL	Upper 95% CL	B_{max} (nM)	Lower 95% CL	Upper 95% CL	n
hot sat	Chapter 3	**3.39**	1.67	6.85	**50**	31	78	3 pools
cold sat	Chapter 4	**3.91**	1.65	9.30	**32.6**	25.1	42.4	8 (mix)
hot sat	vitellogenic [1]	**3.81**	2.86	5.07	**60**	50	71	5 fish
cold sat	vitellogenic [1]	**3.90**	1.17	12.98	**47.4**	32.8	68.4	5 fish
hot sat	non-reprod [1]	**3.05**	2.26	4.10	**39**	33	46	5 fish
cold sat	non-reprod [1]	**3.90**	1.93	7.89	**36.6**	29.3	45.6	5 fish
hot sat	unstressed 1h [2]	**3.23***	-	-	**53.1***	-	-	5 fish
hot sat	stressed 1h [2]	**3.14***	-	-	**49.7***	-	-	5 fish
hot sat	unstressed 6h [2]	**3.49***	-	-	**57.2***	-	-	6 fish
hot sat	stressed 6h [2]	**2.84***	-	-	**49.5***	-	-	6 fish
hot sat	unstressed 24h [2]	**3.97***	-	-	**55.3***	-	-	5 fish
hot sat	stressed 24h [2]	**3.40***	-	-	**54.2***	-	-	5 fish

[1] Chapter 5.

[2] Chapter 6.

* means calculated from assays analysed individually in LIGAND.

Table 7.3: Estimates of k_D and B_{max} and associated 95 % confidence limits from all assays of snapper plasma.

Method	Sample	k_D (nM)	Lower 95% CL	Upper 95% CL	B_{max} (nM)	Lower 95% CL	Upper 95% CL	n
hot sat	Chapter 3	**10.7**	6.8	16.8	**39**	27	55	3 pools
cold sat	Chapter 4	**18.7**	2.2	158.3	**23**	14	38	5 (mix)

Table 7.4: Estimates of k_D and B_{max} and associated 95 % confidence limits from all assays of greenback flounder plasma.

Method	Sample	k_D (nM)	Lower 95% CL	Upper 95% CL	B_{max} (nM)	Lower 95% CL	Upper 95% CL	n
hot sat	Chapter 3	**85**	55	132	**96**	70	133	1 pool
cold sat	Chapter 3	**85**	56	128	**164**	124	216	3 pools
cold sat	Chapter 4	**77**	23	254	**284**	215	375	7 (mix)
cold sat	vitellogenic [1]	**227**	134	384	**376**	255	554	5 fish
cold sat	non-reprod [1]	**239**	147	390	**407**	283	585	5 fish

[1] Chapter 5.

of E_2 from flounder SBP (Chapter 3, this volume), where a slight difference in the slope between assays will have a disproportionate effect on k_D and B_{max}. Differing rates of dissociation in the different species (Chapter 3, this volume) (and also different dilutions used in the assays) suggested that assays from the 4 species were best not compared to each other. However, differences in binding characteristics appear to exist beyond any artefactual differences from this method (Chapter 3, this volume).

To determine whether SBP levels were related to peak plasma levels of steroids, binding characteristics of SBP were measured in trout, bream, snapper and flounder and compared (Chapter 3, this volume). Differences in k_D, B_{max} and rates of dissociation of E_2 from SBP were found between rainbow trout, black bream, greenback flounder and snapper. Contrary to initial expectations, these differences in binding affinities and capacities between species were not strongly correlated with peak plasma levels of E_2 and T (Chapter 3, this volume). Nor were the differences entirely attributable to the general trend of changing k_D and B_{max} with phylogeny (Callard and Callard, 1987). The trend was followed by rainbow trout and the sparids (bream and snapper), with estimates of both k_D and B_{max} higher in the more phylogenetically primitive salmonid. However, the binding characteristics of flounder did not follow this trend. It is possible that flounder, or perhaps the pleuronectidae may be an exception to the trend. Alternatively, it is more likely that the trend of decreasing B_{max} in more phylogenetically modern species may only be valid at a much broader level. Differences in binding characteristics may be obscured or overemphasised depending on a number of assay variables including the method of separation of bound and free steroid, the time for this separation, and the plasma dilution factor (Chapters 2 and 3, this volume). There is also inherent difficulty in comparisons of binding characteristics between studies as evidenced by the widely differing estimates of k_D and B_{max} for rainbow trout between the present study (k_D = 0.44 nM, B_{max} = 92 nM) and previous reports by Fostier and Breton, (1975; k_D = 5.55 nM, B_{max} = 2000 nM), Foucher *et al.* (1992; k_D = 2 - 3.3 nM, B_{max} ~ 400 nM) and Pottinger and Pickering (1990; k_D = 16.8 nM, B_{max} = 2136 fmol.mg protein^{-1}). However, even allowing for these considerations, differences were still apparent in k_D and B_{max}, and rates of dissociation of E_2 from SBP between the 4 species investigated in the present study. Substantial differences between the binding characteristics of flounder SBP (moderate affinity, high capacity, extremely fast dissociation) and those of rainbow trout, black bream and snapper (higher affinity,

lower capacity, fast dissociation) suggested that the protein measured in flounder may not have been a typical SBP (Chapter 3, this volume).

A distinguishing characteristic of SBP is that it binds a number of steroids. However, as with higher vertebrates, the trend in teleosts is for high affinity binding of predominantly E_2 and/or T (Fostier and Breton, 1975; Westphal, 1986; Pasmanik and Callard, 1986; Pottinger, 1986; Pottinger and Pickering, 1990; Foucher *et al.*, 1991). Other steroids binding with moderate to high affinity to teleost SBP include dihydrotestosterone (DHT), androstenedione (A), estrone (E_1) and 11-ketotestosterone (11-KT) (Fostier and Breton, 1975; Pasmanik and Callard, 1986; Pottinger, 1986; Pottinger and Pickering, 1990; Laidley and Thomas, 1994). Xenobiotic estrogens can also bind to SBP but only displace endogenous steroids at very high concentrations (Milligan *et al.*, 1998). Despite differences between the binding characteristics of flounder SBP, and those of the SBP in trout, bream and snapper, the rank order of binding affinity of a range of related steroids was similar in all 4 species (Chapter 4, this volume). The rank orders of affinity for all 4 species were also similar to results reported from other fish (eg. Pasmanik and Callard, 1986; Pottinger, 1986; Laidley and Thomas, 1994). Thus the proteins measured here by specific binding of E_2 in trout, bream, snapper and flounder were confirmed as SBPs. Rank orders of affinity found in this study were also similar to those of SBP in other vertebrates (Westphal, 1986), confirming that the structural requirements necessary for the steroid molecule to bind with high affinity to SBP (a planar steroid nucleus, an unhindered 17β-hydroxyl group and a carbonyl or hydroxyl at C-3; Petra, 1991) appear to have remained constant throughout evolution.

The estimates of k_D and B_{max} for SBP in male and female fish of the same species tend to be similar (Pottinger, 1986; Laidley and Thomas, 1997). This similarity of binding characteristics was confirmed for male and female rainbow trout in the present study (Chapter 5, this volume). Although sex differences in binding characteristics appear to be minimal, levels of SBP do change with reproductive stage in males (Pottinger, 1988; Foucher *et al.*, 1992) and females (Laidley and Thomas, 1997) of some species, but differences are absent from other species (Pasmanik and Callard, 1986; Chang and Chen, 1990b, 1991). There was a significant increase in B_{max} in vitellogenic females of both trout and bream, compared to non-reproductive fish (Chapter 5, this volume) that was of similar

magnitude to that found in spotted seatrout (*Cynoscion nebulosus*; Laidley and Thomas, 1997). Differences in B_{max} with female reproductive stage are not present in all species (Pasmanik and Callard, 1986; Chang and Chen, 1991), and in support of this fact, no differences in B_{max} with reproductive stage were found in flounder in the present study (Chapter 5, this volume). The increase in B_{max} during the vitellogenic stage of some female fish (Chapter 5, this volume; Laidley and Thomas, 1997) may allow continued action of SBP as an effective buffer in the presence of elevated plasma steroid levels (Mendel, 1989). However, the importance of increased B_{max} is questionable as differences in B_{max} with female reproductive stage are not ubiquitous (Chapter 5, this volume; Pasmanik and Callard, 1986; Chang and Chen, 1991). Competitive inhibition of E_2 binding resulting in reduced affinity of E_2 for SBP in trout may result from high plasma levels of vitellogenin (Chapter 5, this volume). However, this could also be an effect of polyunsaturated fatty acids, which are elevated in mature fish (Ballantyne *et al.* 1996) and act competitively to reduce the affinity but not capacity of goldfish SBP (Van Der Kraak and Biddiscombe, 1999).

There is evidence that stress may alter SBP levels through the action of cortisol (Pottinger and Pickering, 1990). The hypothesis that SBP levels may be reduced by stress and thus provide a mechanism for the rapid and significant decrease of plasma E_2 and T levels seen in a variety of species (Carragher and Pankhurst, 1991; Pankhurst and Dedual, 1994; Haddy and Pankhurst, 1999) was investigated (Hobby *et al.* 2000; Chapter 6, this volume). While a profound effect was not present in the bream and trout investigated, the reductions in B_{max} observed may comprise a component of the stress response. Reduced plasma levels of SBP would lower the efficacy of SBP as a reservoir of steroids. There is also significant competition for SBP binding sites in bream, but not trout plasma, by physiological concentrations of cortisol. It is possible that elevated levels of cortisol may reduce the amount of E_2 and/or T bound to bream SBP. This would result in increased metabolism of these sex steroids, and their removal from the plasma.

In conclusion, the presence of SBP has been confirmed in the 4 species investigated in the present study. Differences in k_D, B_{max} and rates of dissociation are present between the 4 species. There is an increase in B_{max} in vitellogenic compared to non-reproductive trout and bream, but not in flounder. It also appears that stress, probably through the action of

cortisol, may reduce B_{max} in trout and bream, and competition of physiological concentrations of cortisol for SBP binding sites in bream may contribute to the reductions in plasma levels of E_2 and T associated with stress. Results from the present study predominantly support the idea of SBP as a steroid reservoir, protecting and transporting E_2 and/or T to target tissues. It would be interesting to discover whether receptors for SBP exist on cell membranes in any lower vertebrates. The most recent advances in understanding the functions of human SBP suggest that the conventional mechanism of intracellular binding of steroids to receptors may only be a part of the story.

7.1 References

Ballantyne, J.S., Mercure, F., Gerrits, M.F., Van Der Kraak, G., McKinley, S., Martens, D.W., Hinch, S.G., and Diewert, R.E. (1996). Plasma nonesterified fatty acid profiles in male and female sockeye salmon, *Oncorhynchus nerka*, during the spawning migration. *Can. J. Fish. Aquat. Sci.* **53**, 1418-1426.

Baulieu, E.E. (1986). Steroid hormone binding proteins and their intra- and extra-cellular congeners. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 1-11. John Libbey Eurotext, London.

Callard, I.P., and Callard, G.V. (1987). Sex steroid receptors and non-receptor binding proteins. *In* "Hormones and Reproduction in Fishes, Amphibians, and Reptiles" (D.O. Norris, and R.E. Jones, Eds.), pp. 355-384. Plenum Press, New York.

Carragher, J.F., and Pankhurst, N.W. (1991). Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). *In* "Reproductive Physiology of Fish 1991" (A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe, Eds.), pp. 253-255. FishSymp91, Sheffield.

Chang, C.F., and Chen, M.R. (1990a). The difference in degree of steroid-binding activity in fishes. *Bull. Inst. Zool., Academia Sinica* **29**, 41-47.

Chang, C.F., and Chen, M.R. (1990b). Fluctuations in sex steroids and sex steroid-binding protein during the development and annual cycle of the male common carp, Cyprinus carpio. *Comp. Biochem. Physiol.* **97A**, 565-568.

Chang, C.F., and Chen, M.R. (1991). Lack of correlation found between plasma sex steroids and sex steroid-binding capacity during the annual cycle of the female common carp. *Bull. Inst. Zool., Academia Sinica* **30**, 81-86.

Frairia, R., Fortunati, N., Fissore, F., Fazzari, A., Zeppegno, P., Orsello, M., and Berta, L. (1992). The membrane receptor for sex steroid binding protein is not ubiquitous. *J. Endocrinol. Invest.* **15**, 617-620.

Fostier, A., and Breton, B. (1975). Binding of steroids by plasma of a teleost: the rainbow trout, *Salmo gairdnerii*. *J. Steroid Biochem.* **6**, 345-351.

Foucher, J.L., Niu, P.D., Mourot, B., Vaillant, C., and Le Gac, F. (1991). *In vivo* and *in vitro* studies on sex steroid binding protein (SBP) regulation in rainbow trout (*Oncorhynchus mykiss*): influence of sex steroid hormones and of factors linked to growth and metabolism. *J. Steroid Biochem. Molec. Biol.* **39**, 975-986.

Foucher, J.L., Le Bail, P.-Y., and Le Gac, F. (1992). Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **85**, 101-110.

Haddy, J.A., and Pankhurst, N.W. (1999). Stress-induced changes in concentrations of plasma sex steroids in black bream, *Acanthopagrus butcheri*. *J. Fish Biol.* **55**, 1304-1316.

Hammond, G.L. (1995). Potential functions of plasma steroid-binding proteins. *Trends Endocrinol. Metab.* **6**, 298-304.

Hobby, A.C., Pankhurst, N.W., and Haddy, J.A. (2000). The effects of short term confinement stress on binding characteristics of sex steroid binding protein (SBP) in female black bream (*Acanthopagrus butcheri*) and rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology* **125A**, 85-94.

Hryb, D.J., Khan, M.S., Romas, N.A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048-6054.

Laidley, C.W., and Thomas, P. (1994). Partial characterization of a sex-steroid binding protein in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **51**, 982-992.

Laidley, C.W., and Thomas, P. (1997). Changes in plasma sex steroid-binding protein levels associated with ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). *Biol. Reprod.* **56**, 931-937.

Martin, M.E., Vranckx, R., Benassayag, C., and Nunez, E.A. (1986). Modifications of the properties of human sex steroid-binding protein by nonesterfied fatty acids. *J. Biol. Chem.* **261**, 2954-2959.

Mendel, C.M. (1989). The free hormone hypothesis: a physiologically based mathematical model. *Endocr. Rev.* **10**, 232-274.

Milligan, S.R., Khan, O., and Nash, M. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (*Oncorhynchus mykiss*) plasma. *Gen. Comp. Endocrinol.* **112**, 89-95.

Pankhurst, N.W., and Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *J. Fish Biol.* **45**, 1013-1025.

Pankhurst, N.W., and Van Der Kraak, G. (1997). Effects of stress on reproduction and growth of fish. *In* "Fish Stress and Health in Aquaculture" (G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, Eds.), pp. 73-93. Cambridge University Press, Cambridge.

Pasmanik, M., and Callard, G. (1986). Characteristics of testosterone-estradiol binding globulin (TEBG) in goldfish serum. *Biol. Reprod.* **35**, 838-845.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Molec. Biol.* **40**, 735-753.

Pottinger, T.G. (1986). Estrogen-binding sites in the liver of sexually mature male and female brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **61**, 120-126.

Pottinger, T.G. (1988). Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **70**, 334-344.

Pottinger, T.G., and Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **80**, 264-273.

Rosner, W. (1990). The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocr. Rev.* **11**, 80-91.

Rosner, W., Hryb, D.J., Khan, M.S., Nakhla, A.M., and Romas, N.A. (1992). Sex hormone-binding globulin: Binding to cell membranes and generation of a second messenger. *J. Androl.* **13**, 101-106.

Rosner, W., Hryb, D.J., Khan, M.S., Nakhla, A.M., and Romas, N.A. (1999). Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. *J. Steroid Biochem. Molec. Biol.* **69**, 481-485.

Stanczyk, F.Z., Namkung, P.C., Fritz, M.A., Novy, M.J., and Petra, P.H. (1986). The influence f sex steroid-binding protein on the metabolic clearance rate of testosterone. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 555-563. John Libbey Eurotext, London.

Van Der Kraak, G. (1983). An introduction to gonadotropin receptor studies in fish. *In* "Fish Physiology IXA" (W.S. Hoar, D.J. Randall, and E.M. Donaldson, Eds.), pp. 405-441. Academic Press, New York.

Van Der Kraak, G., and Biddiscombe, S. (1999). Polyunsaturated fatty acids modulate the properties of the sex steroid binding protein in goldfish. *Fish Physiol. Biochem.* **20**, 115-123.

Vermeulen, A. (1986). TeBG and CBG as an index of endocrine function. *In* "Binding Proteins of Steroid Hormones" (M.G. Forest, and M. Pugeat, Eds.), pp. 383-396. John Libbey Eurotext, London.

Westphal, U. (1986). "Steroid-Protein Interactions II", p. 592. Monographs on Endocrinology 27, Springer-Verlag, New York.